

Support small private telescopes

The need to prioritize is focusing US astronomers' attention on threats to national facilities. But there are many smaller optical telescopes run by universities that represent an underused resource.

To astronomers, closing down a telescope can be a traumatic experience — a bit like losing an old friend. But it is the threat to their science that underlies astronomers' concern, in the United States in particular, that smaller instruments are at risk from budget cuts. Nevertheless, given the current fiscal constraints faced by the National Science Foundation (NSF) and the national observatories, changes in the way astronomical research is practised in the United States are inevitable.

The most important qualities of the national observatories seem unlikely to change. In particular, the National Optical Astronomy Observatories (NOAO) and the NSF are both committed to providing a suite of telescopes at Kitt Peak in Arizona. More contentiously but laudably, NOAO will also continue to support visiting researchers who are not established optical astronomers but who require optical data to complement those from other wavelengths.

Much good research remains to be done on small telescopes; for example, smaller instruments tend to be the most efficient way of studying large patches of sky or extended objects, carrying out surveys and monitoring variable objects. But much of that work might better be carried out at universities rather than at the national observatories. True, some research programmes are appropriate for the latter sites, such as observations using large-format charge-coupled-device cameras; such detectors are beyond the budgets of most university observatories. But a network of university-owned and operated 1-metre-class telescopes might even lead to an increase in the total amount of observing time available. That would be especially so if coupled with the construction of a new 2.5-metre telescope at Kitt Peak itself.

Astronomers are justifiably concerned that university observatories provide little support for outside users, little documentation about the software needed to run telescopes, and — often — poor cameras or spectrographs. Private observatories are cheaper to run than the small telescopes operated by NOAO, but only because they sacrifice the characteristics that make NOAO so useful. Before astronomers accept gracefully the changes being forced on them, those legitimate concerns will have to be addressed.

The national observatories exist to provide competitive access to the best facilities available, and priorities have correctly been chosen to maximize value for money. The new Gemini telescopes in Hawaii and Chile make savings necessary elsewhere, given the large number of small telescopes in the United States; the main challenge facing optical astronomers is not how to cope with an imminent lack of facilities but how to allocate resources in a way that makes best use of all that is available.

To that end, NOAO needs to complete an inventory of private observatories, and to assess their effectiveness. The best could be offered technical assistance by NOAO and financial assistance by the NSF and the National Aeronautics and Space Administration to upgrade their facilities. In return, those observatories would set aside a proportion of their time for visiting astronomers, and provide software similar to that used at the national observatories. NOAO already has the appropriate software; the private observatories need provide only an interface between their control programs and the NOAO software. NOAO should handle the allocation of observing time, ensuring even-handed access.

Achieving this will require some astronomers to revise their expectations. To ease that transition, NOAO and the NSF must continue to reassure the astronomical community that they remain committed to providing the facilities, especially at Kitt Peak, necessary to do top-notch research. And if the whole community is to achieve its common goals — a productive and cost-effective policy on telescope facilities — the lamentable communication gap between the senior levels of NOAO and the NSF and the grassroots astronomers must be closed. □

Tenured hypocrisy

Employment policies in Japan's national universities are indefensible and amount to racial discrimination.

TENURE is a privilege that all faculty members of Japan's 98 national universities are granted automatically and irrevocably — provided they are Japanese. That situation embodies two distinct scandals: excessive cossetting of tenured academics alongside institutionalized discrimination, sometimes arbitrarily practised, against foreigners. Regrettably, that indefensible position is set to persist. An attempt by reformers to introduce limited-term appointments for Japanese is being fiercely resisted by the academic community (see page 654), whereas the same community shows great reluctance to grant tenure to non-Japanese.

In 1982, Japan revised its laws to allow non-Japanese to be employed at national universities on the same terms as local nationals — with one significant difference. Reformers had intended that foreigners should get tenure, but that was resisted by conservative politicians and by powerful university officials, who won the battle. The regulations, as a result, state that "in principle foreign faculty should be hired on a temporary basis, but, in unavoidable circumstances" the granting of tenure to foreign faculty "is not illegal" (see *Nature* **345**, 380; 1990).

Not surprisingly, 14 years after the introduction of the law, employment figures are remarkably unbalanced. There are only 66 non-Japanese with tenure, compared with more than 35,000 Japanese, and more than 85 per cent of the 461 foreigners employed under the new system (as of July 1995) are on limited-term contracts. Furthermore, there is no system of assessment to decide which non-Japanese get tenure. That depends on the decision of the individual university and department head.

The situation of foreign nationals employed under the old system that existed before 1982 (and which still exists today) is even worse. They are all on one-year contracts. And, as some of the more senior ones found out recently to their dismay, they can be dismissed at the wave of a hand from the education ministry.

Japanese university and government officials have made much play over the past decade of the need to internationalize the public research sector. But what self-respecting foreign scientist would want to seek employment in present circumstances? Japan needs to introduce a combination of limited-term and tenured positions that allows only young academics who have proved their worth, be they foreign or Japanese, to obtain permanent employment □



Researchers resist copyright laws that could endanger data access

Washington. Researchers across the world could lose access to important databanks under new database copyright rules being considered by the World Intellectual Property Organisation (WIPO), scientific leaders in the United States warned last week.

The critics say that, in order to use data under the proposed rules, astronomers, climatologists, oceanographers and others who rely on free access to large observational databases would have to seek permission and perhaps to pay. The proposed rules are said to have originated in Europe and have already been endorsed by the US Department of Commerce.

But US government lawyers say that the concerns are misplaced. According to one of them, Keith Kupferschmid of the US Patent and Trademark Office (PTO), scientists will get free access to data anyway under the proposed law, because their using it will not do "substantial harm" to the commercial interests of the database compiler.

Leaders of the US scientific establishment have written to Michael Kantor, secretary of commerce, criticizing the proposed new rules. They want the United States to withdraw its support and to prevent their endorsement at a planned WIPO meeting in Geneva in December.

In the letter, Bruce Alberts, president of the National Academy of Sciences, William Wulf, president of the National Academy of Engineering, and Kenneth Shine, of the Institute of Medicine, say that the proposed changes would "significantly inhibit researchers seeking to reuse and combine data for publication or for research".

The changes were endorsed by the commerce department "without any debate or analysis of the law's potentially harmful implications" for science and technology, the letter says. It points out that, although the unintended consequences appear "very grave", no effort was made to consult the scientific community.

The proposals would assign the copyright on the contents of a database to whoever compiled it, require users to ask for permission to use the contents, and set up a basis for payment for use. Critics say that the law would apply to all privately generated datasets, as well as to public datasets from countries that wished to restrict access.

Climate scientists are particularly worried that cash-strapped weather services in some countries could use the rule to extract fees from researchers who need access to their weather records. According to John Barton of the law school at Stanford University, the

privatization of satellite and other data-gathering operations will place an increasing quantity of vital data in the hands of organizations that will be inclined to use the new law to charge scientists for access. "The entire community ought to be very upset about this," says Wulf.

Stephen Berry, a chemist at the University of Chicago, attacks the proposal for failing to include any "fair use" provision of the type that currently allows free use of documents and databases for research. In the worst scenario, he says, the proposal would allow a commercial database compiler to "co-opt" publicly accessible data and claim copyright on it.

According to Berry, the proposal won the backing of the United States because commercial publishers exerted their influence before scientists became aware of what was happening. He now hopes that pressure from scientists can prevent the WIPO meeting from endorsing the plan.

Scientists have been alerted to the issue by the computer data committee of the International Council of Scientific Unions,

and are pushing for changes to protect the principle of free access to data for research.

Bruce Lehman, commissioner of the PTO, was due to meet Alberts, Wulf and Shine this week to discuss their concerns. Kupferschmid of the PTO says that databases will be protected only where a substantial investment has been made and the use would substantially harm that investment. He adds that "most of US commerce and industry is happy with the proposal".

WIPO will meet for three weeks in December to consider three new international treaties to protect databases, literary and artistic works and music recordings respectively. Each treaty will require ratification by signatories — by the Senate, in the case of the United States.

But the academies' letter to Kantor warns that anything adopted by WIPO "will move substantially toward becoming the new international norm" by the end of this year — regardless of US Senate ratification. The PTO will hold a forum to discuss the draft proposals in Arlington, Virginia, on 12 November.

Colin Macilwain

Massive star comes into bright focus

London. The orbiting Hubble Space Telescope has captured rare images of a giant star (right), which is 30 to 60 times as massive as the Sun and whose brightness has increased 40-fold in the past three years. The star belongs to a class known as erupting luminous blue variables (LBV) and is still in a very unstable and eruptive period of its life.

The star is the largest of a cluster of erupting stars and luminescent gases distributed in the shape of a fish-hook. All are contained in the Magellanic galaxy NGC2366, about 10 million light years from Earth.

The Hubble image shows two further dense clusters of massive stars. The older cluster at the top of the picture is around 4 to 5 million years old. It is relatively dim, having lost its luminescent gases to the effects of stellar 'winds' and supernova blasts. The younger and brighter cluster (bottom centre) is probably less than 2 million years old.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
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Only four LBV eruptions have been recorded. This latest eruption was first spotted at the beginning of the year. The rate of increase in its brightness was measured by comparisons with previous ground-based images. □

Hubble Space Telescope

Japan's academics fight erosion of tenure...

Tokyo. Proposals intended to introduce limited-term employment and the assessment of research performance for university faculty members in Japan are meeting strong resistance from universities. As a result, although the labour laws will probably be revised to implement the proposals, such contract employment is unlikely to be widely adopted in the near future.

Earlier this month, a subcommittee of the University Council, an advisory body to the Ministry of Education, Science, Sports and Culture (Monbusho), approved a draft plan calling for the reform of employment practices. The proposal, likely to be adopted by the council and the ministry next week, follows suggestions for reinvigorating the public-sector research system outlined in the five-year plan for science and technology

adopted this year by the Japanese cabinet.

The reforms are revolutionary for Japan, where all university faculty members — with the exception of non-Japanese (see below) — are government employees who enjoy guaranteed jobs for life, regardless of their academic performance.

But the proposals, which call for application of limited term employment at all faculty levels up to and including professors, have met strong resistance from professors within and outside the council. Akito Arima, president of the Institute of Physical and Chemical Research and chairman of the subcommittee that drafted the proposals, admits that concessions have had to be made as a consequence.

It is now being recommended that individual universities should decide whether to

adopt the new practices. There will be "no strong recommendation from the government", says Arima. But he is pleased that, provided the plan is adopted next week, Monbusho will amend the laws for the employment of university faculty members to allow for contract employment, even if it takes "five to ten years, or even a hundred years" for significant change.

Arima hopes that contract employment will be used for young staff entering the system. Over recent years, the government has strengthened the graduate schools of many universities and, as a result, many research associates (*joshu*), have been promoted to associate professor or professor. But, because of a ceiling on the number of full-time government jobs, the *joshu* positions have been left unfilled. Arima's intention is that these positions should be filled with contract employees.

The subcommittee recommends that, at the end of the contract period, the employees should move to another university. They should be allowed to stay at the first institution for another term only in exceptional circumstances and after "very severe review" of their performance, says Arima. The subcommittee wants to encourage employees to move between Japan's universities, many of which suffer from 'in-breeding'.

But again, it will be left to individual institutions to decide whether to adopt these recommendations. Many university faculty members are opposed to the assessment of the research performance of individuals. They claim that it is hard to establish fair criteria and that such assessment will encourage short-term research with quick results, undermining longer-term work.

Another criticism of the reforms is that they take no account of teaching performance. Faculty members who are good teachers but do little research will be penalized. But Arima argues that the reforms are aimed chiefly at young researchers who do little teaching. In any case, he says, universities could start to assess teaching performance if they wish.

David Swinbanks

... as foreign staff fear greater insecurity

Tokyo. Japanese faculty members in Japan's national universities enjoy tenure — and many are resisting the introduction of limited-term contracts — but most foreign academic staff are already on limited-term contracts.

The few hundred foreign academic staff in Japan are already feeling the pressure of government attempts to encourage universities to employ younger staff. They fear that adoption of the University Council's proposals for limited-term employment (see above) could make their situation worse, as the recommendations also apply to private universities, which have until now employed foreign faculty members on a long-term basis.

There are two systems for employment of foreign faculty members in Japan. Under the *kyoshi* system, which dates back to the Meiji era in the last century, foreign teachers are employed on one-year contracts with relatively high pay. It is senior people in this category who have recently been fired.

As an alternative, in 1982 the government introduced the *kyoin* system, under which foreigners are hired as regular department members on a longer-term basis, but on the same pay scale as Japanese colleagues. Even under this system, however, most foreigners are on limited-term contracts, averaging three years. Only a few — 66 out of 461 in July 1995 — have been given tenure.

The situation for foreigners who have worked in Japanese universities for many years recently became even more insecure. A 1992 directive from the Ministry of Education, Science, Sports and Culture (Monbusho) advised universities,

in the interests of the national economy, to employ young foreign faculty members whenever possible because of their lower salaries. This led to the dismissal of tens of senior foreign faculty members between 1993 and 1996. Many of them had been approaching eligibility for retirement pensions.

The plight of several senior foreign *kyoshi* who were dismissed following the directive was raised with Monbusho by Walter Mondale, the US Ambassador to Japan, earlier this year. The issue was later discussed in the Diet (Japanese parliament). But the ministry's response, in a directive to universities last June, was merely to repeat its advice that universities should, wherever possible, hire younger people.

The only concession made by the ministry is its suggestion that, in considering the renewal of contracts, age should not be the only criterion, and that it should be emphasized to new employees that they are only on one-year contracts, with no guarantee of long-term employment.

Ivan Hall, a Japan historian and one of those dismissed, describes the treatment of foreign faculty members as "academic apartheid". He sees little hope of improvement, and says that the root of the problem lies with Japanese faculty members who are reluctant to employ foreigners on a long-term basis because of a deep-rooted fear that they will be disruptive to the existing system. This is despite repeated calls over the past decade by university and government officials for greater "internationalization" of Japan's universities and its public research system. **D. S.**

Dengue fever in Delhi

New Delhi. An epidemic of dengue haemorrhagic fever (DHF) sweeping New Delhi, the capital of India, has killed 200 people in six weeks and at least 4,000 are in hospital. With eight to ten deaths being reported daily, the epidemic has created panic among those in whom memories of 1994 plague (see *Nature* 372, 119; 1994) are still fresh.

"The problem is more serious than plague," says N. P. Gupta, former director of the National Institute of Virology. "We had very good drugs for plague, but there is nothing specific against DHF." □

Aboriginal relations strained by theft of dinosaur footprints

Sydney. Thieves have sawn rare fossilized dinosaur footprints out of rocks in Western Australia, angering palaeontologists who had been hoping to identify the animals — possibly stegosaurs — that left the marks.

The theft has caused bitterness in the local Rubibi Aboriginal community, which has regarded the area as sacred for generations. The site is on a foreshore near Broome in the north of the state. The fossil-bearing rocks were first examined by scientists in 1991, following the promotion by the Western Australian Museum in Perth of a public "great dinosaur hunt".

The area is rich in footprints of brontosaurus, other herbivores and predator dinosaurs. But Tony Thulborn, a palaeontologist at the University of Queensland, says the stolen footprints were "different and very rare". The prints of five-fingered hands are about 20 cm wide and somewhat shorter in length; the three-toed hind feet are 20 cm long and narrower in width.

Thulborn says the early Cretaceous sediment in the region is thought to be between 114 million and 120 million years old, and reveals what he describes as a "treasure trove" of diverse fauna walking on a forest floor. Confirmation that stegosaurs lived in Australia would reinforce the inclusion of the Australian continent as part of the southern super-continent of Gondwana at that time.

But Thulborn challenges claims that the fossil footprints are sufficiently distinct for unambiguous identification as stegosaurs, which would give them historical precedence over other unpublished claims, mainly from North America. Given the limited studies so far, he says that he can categorize the animals that left the prints only as thyreophorans, a group of armoured, herbivorous dinosaurs that includes stegosaurs and ankylosaurs.

Following the 'hunt', mostly by amateurs, Thulborn says it took five years for him to establish confidence among the Aborigines to gain access to the site for detailed, professional studies of the footprints. He claims that the theft of the specimens is "not scientifically devastating" as casts and photos had been taken. But he adds that "the real loss is in understanding of our work among the local community which holds that sacred sites should be seen only by initiated males".

Publication of pictures in the media has not helped to capture the thieves. It seems unlikely that professional collectors were involved, as the museum and black markets are profitable more for large skeletons than for footprints, which are not easy to see.

Peter Pockley

Max Planck institutes 'could still be saved from closure'

Munich. At least one of four research institutes run by Germany's Max Planck Society (MPS) that were unexpectedly earmarked for closure earlier this month could be saved, according to Hubert Markl, president of the society.

But this will require the government to modify its so-called 'federal consolidation programme' under which all publicly funded institutes are to shed 2 per cent of their workforce annually to the end of the decade (see *Nature* 383, 566; 1996).

According to Markl, Jürgen Rüttgers, the federal research minister, has told him informally that the programme will be extended beyond 1998 — its approved lifespan — to at least 2000, and that the Max Planck Society will not be exempt. Markl says this is why it became necessary to announce the closures. But he says that the best chance of the institutes' survival is for politicians to be sufficiently moved by the seriousness of the situation to exempt the society as a whole from the federal consolidation programme.

The MPS has long been warning that closures were likely in the west of the country if its budgetary squeeze continued. Markl

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Markl: fighting cuts.

Stefan Hülzer

says that the principle had been widely acknowledged. But now that it has become a reality, and he has thrown the names of four west German institutes into the arena, reactions have been hostile. "I expected some president-bashing," he says.

The four institutes destined for closure — two in biology, one in history and one in space science — are asking their respective regional (*Länder*) governments and scientific communities to support their attempts to stay open. The Institute for Aeronomy in Lindau has already attracted international support, largely because of its involvement in international planetary missions (see below). The institutes will have time to consider their strategy, as the decision-making procedures are relatively slow.

If the society is not exempted from the federal consolidation programme, the closure of the four institutes will not be the end of the story, as the resulting staff reduction will total only about half of that required. Markl has asked each of the society's three sections — biology, physical sciences and humanities — to suggest six departments for closure, cutting an additional 250 posts.

Axel Korth, a spokesman for the Institute for Aeronomy, says that some researchers are hoping that the MPS will decide to close more departments, to save whole institutes. But this is thought unlikely. All final closure decisions will be taken during next year.

Alison Abbott

Plans cast cloud over Rosetta mission

Munich. Concern is growing about the fate of the European Space Agency (ESA) Rosetta mission to the comet Wirtanen, due to be launched in 2003, following the announcement that the Max Planck Institute for Aeronomy at Lindau, Germany, could be closed within the next few years (see above).

The institute is the base for two of the mission's principal investigators, who are developing a microwave spectrometer and Rosetta's imaging system, called Osiris. The Lindau institute is also principal developer for Rosetta's lander.

The uncertainty is causing substantial concern, says Marcello Coradini, ESA spokesman for Solar System sciences. The payloads will be finally approved next February. But the Lindau scientists will find it hard to attract commitments of funding from national research agencies while their institute's own existence is in doubt, he says.

Hans Balsiger, a Solar System scien-

tist from the University of Bern in Switzerland, calls threatened closure of the institute "totally irresponsible". The Max Planck Society has not considered the international dimension, he says. Balsiger shares with the Lindau institute responsibility for one of Rosetta's instruments, and is also chairman of ESA's science programme committee.

Hubert Markl, president of the society, says that current commitments to Rosetta projects will be fulfilled. One reason for selecting the Lindau institute for closure, he says, is that space research is primarily a federal responsibility.

Markl plans to discuss a possible takeover of the institute by DLR, the National Air and Space Research Centre, with DLR's director general, Walter Kröll, in December. But scientists at Lindau view the possible takeover with alarm, as the centre does not share its tradition of conducting basic research alongside instrument development.

A. A.

R&D protection from GATT rules 'should be abolished'

Washington. Subsidies for research and development could be vulnerable to legal challenge if governments change their international trade agreement in line with a recommendation from an international study endorsed by the US National Research Council (NRC).

The study says governments should drop provisions in the General Agreement on Tariffs and Trade (GATT) that protect such subsidies from charges of being an unfair trade practice.

The current exemptions allow governments in the United States, Japan and Europe to support large technology programmes, free from the threat of a challenge under GATT rules. But the study says that these exemptions encourage other countries to press for exemptions for other forms of spending, such as environmental subsidies.

Its authors warn that the exemptions are ill-defined and likely to encourage abuse by governments that want to circumvent the spirit of the agreement. But their conclusions are already being contested by US administration officials, who claim that the exemptions are needed to protect government-industry collaboration.

The NRC is the operating arm of the National Academy of Sciences and the

Bohannon said he did not think there was a good argument to support its finding. "The origin of the special treatment was the reality that the United States invested more than any other nation in R&D infrastructure," he said. The provision was therefore needed so that corporations "would not be harmed by their cooperation with the government" in various R&D programmes.

The "special treatment" drawn up during the GATT negotiations allows corporations to receive from government sources up to three-quarters of basic research costs, half of applied research costs and one-quarter of development costs, without any danger of a subsidy complaint being accepted by the World Trade Organization, the body set up to administer GATT.

But according to Alan Wolff, a lawyer and co-chair of the international panel that oversaw the study, the special treatment is not needed to protect government-sponsored R&D, and undermines GATT by opening the way to other exemptions.

"Experience indicates that R&D subsidies are rarely the subject of counter-measures because they do not cause harm" to competitors, says Wolff. "We feel that government support for R&D is a good thing but, if injury is caused, that subsidy should be actionable" like any other.

Wolff admits that the R&D exemption could not be removed immediately. But he says this should happen when the GATT agreement is reviewed after five years.

Charles Wessner, the staff director of the study, says that the United States negotiated the R&D exemption in GATT hurriedly, after its negotiators had discovered that earlier versions of the agreement "outlawed our own practices" of research support. The US negotiators "got down a cul-de-sac and couldn't find a way out", he says.

The 200-page study — *Conflict and Cooperation in National Competition for High-Technology Industry* — was conducted jointly by the NRC's board on Science, Technology and Economic Policy and two German research institutions, the Hamburg Institute for Economic Research and the Kiel Institute for World Economics. It was paid for by the German-American Academic Council and ten corporate sponsors, and supervised by a panel of economists, academics and industrialists from six countries.

The study finding highlights a significant contradiction in the trade policies of Europe and the United States. Both are publicly committed to free trade in high-technology goods and services, but wish to pursue technology policies that inject billions of dollars of public money into industrial research each year.

Colin Macilwain

India faces US trade action over failure to amend patent law

New Delhi. An Indo-US dispute over patents, raised by the Clinton administration before the World Trade Organization (WTO), is likely to take a decisive turn in the next few weeks after the failure of attempts at a resolution.

The United States is now considering third-party adjudication. The dispute centres on India's failure to bring its patent law into line with WTO's Trade Related Intellectual Property Rights Agreement (TRIPs). The adjudicators' decision, which could include trade penalties against India, would be binding.

Both India and the United States are members of WTO. Under TRIPs, India is obliged to allow the patenting of food and pharmaceuticals, which are excluded from the 1970 Patents Act. The agreement also includes provisions with effect from 1 January 1995 for the 'black-box' facility of lodging product patent applications. The United States has also alleged that, as a signatory of TRIPs, India must agree to provide "exclusive marketing rights" for product patent applicants.

The Indian government, faced with cross-party opposition to proposed amendments to the Patents Act, has been unable to comply with US demands. An amended version of the act, though passed in 1995 by the Lok Sabha, the lower house of parliament, remains blocked in Rajya Sabha, the upper house.

When the previous Congress party government signed the TRIPs agreement, many politicians expressed reservations about the implications for the prices and availability of vital products, such as medicines and seeds. Matters have not been helped by the filing by US companies for patents on products found in India, including one on a process involving the active ingredient of neem (see *Nature* 377, 95; 1995). But government officials are conscious of the potential damage to trade if India is penalized for not complying with TRIPs.

Sixty days of 'mutual consultations' between the two countries, prescribed under WTO dispute procedures, ended in Geneva last month without result. This opens the way for the United States to ask WTO to take the case to a panel of international adjudicators.

Indian officials are bracing themselves for further US pressure through WTO. "If the United States decides to ask for the WTO dispute settlement panel, the implications could be disastrous for India's international trade," says one official.

K. S. Jayaraman



National Academy of Engineering, and is the body on which the federal government relies most heavily for advice on science and technology matters.

Its advice is rarely disputed by government officials in public. But representatives of the US Commerce Department — which launched the drive for the exemptions during the 'Uruguay round' of trade negotiations — immediately attacked this NRC finding in unusually direct terms.

Mark Bohannon, chief attorney in the office of Mary Good, the under-secretary for commerce, told the NRC panel at a public meeting on 17 October that he "regretted very much" its finding, predicting it would divert attention from the rest of the report.

Industry warms to 'flexible' carbon cuts

London. A study appearing to suggest that, rather than making major and immediate cuts in carbon emissions to combat global warming, governments should make moderate reductions now, followed by sharper reductions later, has become the latest focus of controversy in the debate about man-made climate change.

At a conference on the economics of climate change organized by the oil industry in Paris two weeks ago, the study, published in *Nature* earlier this year (379, 240; 1996), was widely heralded by representatives of the industry as a typical example of a 'flexible' approach to emissions reductions.

This response coincides with an apparent shift in the industry's strategy. Having now acknowledged the need to combat global warming, it is warning that proposed controls on carbon dioxide emissions will be vigorously opposed if they are considered too costly to implement.

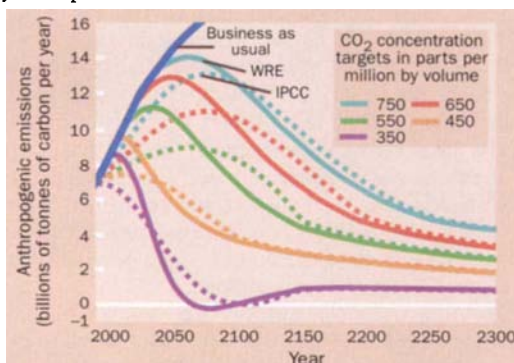
But environmental organizations, and a few industrialists, criticize the paper for appearing to advocate a 'delay or do nothing' policy. Some scientists and economists point out that it represents only one opinion in a controversial field, and should not be used as the sole basis for making policy. Others criticize any efforts to quantify the 'non-market' impact of climate change, such as the effects on biodiversity and the environment.

The conference was organized by the International Petroleum Industry Environmental and Conservation Association, based in London. Its goal appears to have been to counter the effectiveness of the environmentalist movement in dominating the climate change agenda. Organizations such as Greenpeace and the World Wide Fund for Nature have forced the pace of debate since the Earth Summit in Rio de Janeiro five years ago, when they helped to persuade governments voluntarily to reduce carbon dioxide emissions to 1990 levels by 2000.

A new proposed law to curb emissions must be agreed at the next annual conference of the United Nations climate

convention in Kyoto, Japan, in December 1997. This time, the oil industry is keen not to be left behind.

Environmentalists, working in association with the Alliance of Small Island States, are calling for a further 20 per cent reduction in carbon dioxide emissions by 2005. But this is strongly opposed by many developed and developing countries, who fear it could impede their industrialization. It is also



A comparison of IPCC proposals with those of Wigley, Richels and Edmonds (WRE) for stabilizing atmospheric carbon dioxide at five levels. WRE say the least costly option is moderate emissions cuts in the early years followed by sharper reductions later.

opposed by oil-exporting countries, which forecast a drastic reduction in export income, and by the oil industry, whose profits would be reduced.

The oil industry's desire for a flexible approach to emissions reductions — which would have minimal impact on economic growth — appears to be shared by the US administration.

At the Paris conference, the industry marshalled scientific backing for its stance. For example, it drew on evidence presented by Richard Richels of the US Electric Power Research Institute, a co-author of the *Nature* paper that says countries would save money by delaying sharp reductions in emissions.

The paper, whose authors include Tom Wigley, a senior scientist at the US National Centre for Atmospheric Research and an adviser to the UN's Intergovernmental Panel on Climate Change, says that there is

more than one route to stabilizing atmospheric concentrations of carbon dioxide (see figure). The researchers suggest that modest emission reductions early on will give governments time to free up the required extra capital, and to develop lower-cost technologies, and thus to reduce the costs of sharper cuts in carbon dioxide emissions in the future.

Richels reiterated the paper's conclusions at the Paris meeting, but this time using cost-benefit analysis. He produced monetary estimates of the impact on national economies, environment, biodiversity and human health if average global temperatures rose by 2.5 °C following a doubling of atmospheric carbon dioxide concentrations.

He said there were "strong arguments" for concluding that the high costs of an immediate heavy emissions reduction programme might outweigh the potential savings, unless the bulk of 'non-market' damages from climate change occur earlier than expected.

But Michael Grubb, of the Royal Institute of International Affairs in London, says Wigley and Richels have not fully assessed the costs of shifting the burden of emission reductions to a later date. Grubb also questions whether cheaper, less carbon-intensive fuels would emerge without governments providing incentives, which has not yet happened.

Another critic is David Madison of the Centre for Social and Economic Research on the Global Environment in London. Madison says he is concerned that Richels has tried to put values on the damage to biodiversity and the environment from climate change, when there is little expert consensus either on the magnitude or the valuation of potential losses.

Wigley says those who think his *Nature* paper advocates a delay in cutting carbon dioxide emissions "are just plain wrong". Many of his critics have "totally misunderstood the paper". For their benefit, he has written a three-page 'plain language guide'.

Wigley says he is aware that some see a connection between the facts that the paper's conclusions support US policy, and that two of its authors, Richels and Jae Edmonds, work for the US electricity supply industry and the Department of Energy respectively. But he says any suggestions of bias are "extremely inappropriate".

"Both my co-authors are reputable scientists of the highest international standing, who have published extensively in the peer-reviewed literature," he says. "It is not true to say that scientists don't have their individual political biases. Assumptions can be subjective, especially in economics. But these assumptions usually come out in the wash during peer review." **Ehsan Masood**

California drops plan to destroy records

San Francisco. California's Environmental Protection Agency has scrapped a controversial plan to destroy records of dissenting scientific opinions on environmental hazards (see *Nature* 470, 383; 1996).

The agency had ordered scientists to eliminate research data and other records on the health risks of industrial emissions and other pollutants if the documents did not agree with its policy directives. But a public-interest lawsuit prompted officials to suspend and reconsider the policy while the state attorney general's

office reviewed the legal ramifications.

The Natural Resources Defense Council (NRDC), the Environmental Law Center and the Society of Professional Journalists had jointly filed a suit against the agency, arguing that the policy represented bad scientific practice and violated California's laws on open records. "The policy was blatantly illegal," says Al Meyerhoff, senior attorney with the NRDC. The groups are not prepared to drop their lawsuit until they are confident that the policy has never been implemented, he says. **Sally Lehrman**

CJD variant stirs debate on release of data

Paris. British researchers are divided about whether there is a need to change the UK system for reporting cases of Creutzfeldt-Jakob disease (CJD) to provide not only numbers of confirmed cases of a novel variant of the disease as at present, but also numbers of suspected cases.

Although some argue that the early publication of such data would speed up understanding of the transmission of the disease, others fear that the information could create unnecessary panic if handled irresponsibly by the mass media.

The debate coincides with news that a 33-year-old British woman appears to be the fourteenth victim of the new variant, which is known as NV-CJD and was formally identified in March. Those suffering from it are widely considered to have contracted the disease from beef contaminated with the agent that causes bovine spongiform encephalopathy (BSE).

Further evidence to support this hypothesis comes in a paper published this week in *Nature* (see pages 685-690) by a research team headed by John Collinge, of Imperial College School of Medicine at St Mary's Hospital in London, indicating that the biochemical characteristics of the new variant are closer to those of BSE than those of classical CJD.

At present, the UK government releases quarterly updates of the number of 'definite and probable' cases of classic forms of CJD. But it reports only on confirmed cases of NV-CJD, usually following post-mortem neuropathology. Similarly, while the reports give the total number of 'referrals' — suspect CJD cases referred by neurologists to the National Creutzfeldt-Jakob Disease Surveillance Unit in Edinburgh — they say nothing about what proportion of these are suspected of having NV-CJD.

According to Sheila Gore, a senior statistician at the biostatistics unit in Cambridge of the Medical Research Coun-

cil (MRC) and a member of the MRC's committee dealing with human spongiform encephalopathies, such information is essential if epidemiologists are to develop an early idea of changes in the incidence of the disease and of the evolution of any epidemic.

A general consensus exists among European surveillance networks, however, that distinguishing between classic CJD and the new variant among referrals would make sense only if "validated clinical criteria" were available to diagnose the new variant.

In the absence of such criteria, reporting of referrals of suspect NV-CJD cases would be "meaningless", says Robert Will, head of the UK CJD surveillance unit. He adds that the press and public might fail to make the distinction between suspect and confirmed 'cases'. "Say we now had 20 suspect cases," says Will. "It may be that only three have it. To say 20 would be misleading."

Some European scientists say that Will's group does its best to make information available in the political circumstances. One adds that there is also much informal exchange of data between the UK group and other surveillance networks established under the European Union's Biomed research programme. Will himself says: "European collaboration in epidemiology may be the only solution to understanding the new variant of CJD."

Nonetheless, France decided this month to change its reporting system to provide information on suspect cases. Quarterly reports on CJD incidence will also break down the list of referred, and 'probable or confirmed', cases into seven age groups (under 30, over 80, and the intervening five 'decades'). The new reports, the first of which will be published next month, will also emphasize that many of the 'suspect' cases listed may not turn out to be CJD, much less the new variant.

The change to the French system goes further than that which Gore would like to

see introduced in the United Kingdom. She wants a breakdown of referrals to tell how many are under the age of 40. Cases of the new variant mainly arise in this younger age group, she argues (although not exclusively, as NV-CJD has also appeared in older patients), while classic CJD — which usually occurs in older patients — sometimes occurs in patients under 40.

Gore claims that, whereas most referrals have previously involved patients in their sixties, the Edinburgh surveillance unit is probably "increasingly having referrals under the age of 40". Continual monitoring of the number of younger referrals and the proportion subsequently confirmed could provide useful epidemiological information, says Gore. She claims that, if a large proportion of referrals were ultimately confirmed, epidemiologists could use this ratio to make projections based on referrals without having to wait on confirmed cases.

Gore admits that such a system would only add "confusion" if the proportion was as low as 10 per cent. But she is adamant that epidemiologists should have the opportunity to establish the usefulness of data on referred cases. "We need as timely surveillance as possible," she says, arguing that information on suspect cases could provide an "earlier handle" on the evolution of any epidemic.

But Will says that calls to make the number of 'suspect' CJD cases available "reflects naivety about the difficulty of neurological diagnosis". Whereas a sound basis exists for releasing information on the total number of CJD referrals, and the number of 'probable' cases of classic forms of CJD, there is no basis for providing analogous figures specifically for the new variant, he says.

The number of referrals is meaningful for classic forms of CJD, says Will, because the latter can be fairly easily distinguished from other forms of dementia, such as Alzheimer's disease, because of the short duration of the illness and a characteristic electroencephalogram (EEG) that permits a clinical diagnosis in 60 to 70 per cent of patients. But the new variant of CJD is much more difficult to distinguish from other forms of dementia, he says, because the duration of illness is longer and the EEG provides little information. The age of patients is a poor tool for discriminating between CJD and other dementia, says Will.

Will says that the way forward is for his group to publish data on cases of NV-CJD in a peer-reviewed journal as soon as possible. Epidemiologists need not just information on incidence, but also on the age of onset of the disease, the date of referral and the date of death. "This is important epidemiological data that people should have available," he says, adding that publication of the data is imminent.

Declan Butler

French advisors back modified maize

Paris. The body that advises the French government on the testing and sale of genetically modified organisms has restated its support for a bid by Monsanto to import its genetically modified maize into the European Union (EU).

The Commission du Génie Biomoléculaire described the case as an "ideal dossier" where the risks could be clearly assessed. Disagreeing with the official views of other EU countries, the commission said the maize was safe, and should be approved for sale in Europe.

The modified maize contains a gene from the bacterium *Bacillus thuringiensis*

(which makes it resistant to the European corn borer), a herbicide-resistant gene and a marker gene conferring resistance to the antibiotic ampicillin.

The commission dismissed concern that the herbicide-resistant gene might jump to wild relatives of the crop, arguing that maize does not interbreed with other European plants. Claims that the antibiotic-resistance gene might pass to bacteria that are pathogenic to humans and animals were also discounted as "highly improbable" by the commission, which added that such genes are already ubiquitous in ruminant gut flora. **D. B.**

Europe's research chiefs plan joint survey...

Munich. A coordinated study aimed at identifying gaps and weaknesses in Europe's basic research efforts is to be carried out by several European national research-funding bodies. The survey will be the first test of the ability of an informal group known as Eurohorcs (European heads of research councils) to organize a collaborative project.

The survey has been commissioned by the European Science and Technology Assembly (ESTA), the broad-based science advisory body of the European Commission. ESTA wants the survey to consider in particular whether any weaknesses it discovers are detrimental to the well-being and competitiveness of the research community. If so, it will suggest to the commission how such weaknesses could be corrected through the use of European Union funds.

The idea of a broad survey of this type was proposed more than a year ago by Sir Peter Swinnerton-Dyer, a former vice-chancellor of the University of Cambridge and one of ESTA's 100 members. It was not taken up immediately because of the scale of the task, and a less detailed analysis is now being undertaken.

The survey is being organized by Richard Brook, chief executive of the UK Engineering and Physical Sciences Research Council. Brook has divided the task into nine scientific areas, responsibility for which has been divided among Eurohorc organizations. For example, the Italian research council (CNR) will analyse molecular and

cellular biology, Germany's Max Planck Society will consider physics, and the UK Natural Environment Research Council will take on geology.

Representatives of the nine research agencies will meet in Brussels within the next few weeks to draw up a common set of criteria for assessing the research in their respective fields. They will then each set up an international panel to gather information from each country.

Brook has set a tight deadline for completion of the survey, which he says he will present to ESTA by the end of next March. The term of office of the current assembly expires in the middle of next year, when half of its members will be re-elected.

Brook and Jan Borgmann, chairman of

ESTA and formerly head of the Netherlands' National Research Council, both accept that the survey will inevitably be slightly superficial. But Borgmann sees it as a pilot, to be repeated later in more depth.

Brook says that the survey will also be a way of testing the muscle of the Eurohorcs group, which, because of its informal nature and lack of a permanent secretariat, has so far had a low-key presence on the research scene. "It will be a test to see if Eurohorcs can do something effectively and collaboratively," he says, adding that it should also help national governments to set their own research priorities by providing a better understanding of the broader European context in which their research takes place.

Alison Abbott

... as citation 'landscape' takes shape

Washington. The concept of a scientific 'landscape' is being given a literal interpretation by researchers at the Sandia National Laboratory in Albuquerque, New Mexico, who have developed a computer tool that shows up scientific trends as ripples, flows, peaks and gorges on a three-dimensional map.

The 'landscapes' are based on data on the cross-citation of scientific literature compiled by the Institute for Scientific Information (ISI), the Philadelphia-based organization that monitors citations of scientific literature worldwide.

The goal is to provide a way of visualizing the ebb and flow of activity in different science disciplines. Exploration of the landscapes will also identify where cooperation is strong between closely related fields of activity, and where it is weak.

Chuck Meyers is manager of laboratory-directed research at Sandia, much of whose work involves research on nuclear weapons. He says the landscapes could provide new insights for policy-makers, corporations and even intelligence agencies wanting a better understanding of unfolding trends in science.

"People are always asking 'where is the leading edge?' in research", says Meyers, but usually all they have to go on is a mixture of anecdotes and indigestible statistics. "Decision-makers need to be able to look at clear indicators of what's happening and not happening."

Sandia has initially built a landscape for just one field, analytical chemistry, based on 30,000 articles published since 1986. It is now inputting the rest of ISI's physical sciences database — three million articles

Watching the peaks: but where is Silicon Valley?

and their references — into what it calls "a huge supercomputer data matrix" which can be explored using virtual reality.

ISI has been working for years with simple, two-dimensional maps that show how closely different scientific disciplines relate to each other. But mathematicians at Sandia used a mathematical approach based on eigenvectors to make the time taken to run their program proportional to the number of articles, rather than to the square of that number. They then used an advanced virtual-reality tool called Muse to present the information on a computer screen and allow users to explore the landscape at will.

A statement from Sandia says that the idea "originally was conceived to help US intelligence", which is said to have become more active in scientific and industrial espionage as it searches for a post-Cold War role. But ISI hotly denies this.

Meyers says that no intelligence money was received for the project, which was paid for with funds that Sandia is allowed to spend as it wishes on exploratory research. Nevertheless, he says he is now talking to US intelligence about applying the landscapes.

Colin Macilwain

Taiwan finally clears nuclear power plant

Hong Kong. Taiwan's parliament has finally given the go-ahead to a fourth and perhaps final nuclear power plant for the island, after a two-year argument over funding, which has frequently degenerated into public and parliamentary violence. Facing growing public opposition to nuclear power on grounds of safety and cost, the government has suggested that it may drop plans for two more stations, and that Taiwan's substantial energy needs may be met by other forms of power.

The Taiwanese opposition parties had voted against all funding for future nuclear projects, including this one, in May. But on 18 October a cabinet-led appeal vote was passed, as the opposition could not muster the two-thirds majority needed to block the appeal. The US\$6.7-billion station, 60 km north of Taipei at Kungliao, will be built by General Electric of the United States, and will use two Japanese boiling-water reactors, Japan's first nuclear reactor exports. Construction work is expected to begin this week and the plant should be on-stream by 2003.

Lis Tacey

IMAGE
UNAMAGABLE
FOR A COYALGET
FOREASORIG
REASONS

German space centres are set on course for merger

Munich. The German Space Agency (DARA) is almost certain to merge with the national air and space research centre, Deutsche Forschungsanstalt für Luft- und Raumfahrt (DLR), based in Cologne, by the end of next year. Officials are also keen to increase the proportion of Germany's space expenditure that goes on national activities.

Last week, the heads of both organizations presented plans for the merger, as requested by Jürgen Rüttgers, the federal research minister, in July (see *Nature* **382**, 386; 1996). The new organization will be called Deutsches Forschungs- und Managementzentrum für Luft- und Raumfahrt, although it will keep the abbreviation DLR. It will combine the management of Germany's national and international space programmes with its own research and development activities. □

Dangers of 'contracting out'

London. Scientists working for the British government claim that the recent crisis over bovine spongiform encephalopathy (BSE) has underlined the danger of depriving government departments of scientific expertise by contracting out their activities to the private sector. The warning comes in a submission presented last week by the Institution of Professionals, Managers and Specialists (IPMS), the labour union that represents many government scientists, to the House of Commons Select Committee on Science and Technology.

IPMS says it is "vital" that the government does not make any major organizational changes to the way in which publicly funded research is managed in areas such as agriculture before the lessons of BSE are fully evaluated. It also says the BSE crisis has demon-

strated "that science must be an integral part of the decision-making process by politicians, senior decision makers and the public". □

Nobel laureates visit Washington

Washington. The five new US Nobel prizewinners in physics and chemistry visited Washington DC last week to express their concern about the state of basic research, and to pledge to lobby Congress against threatened funding cuts. They criticized Congress and the administration for "knowing very little" about science, and ignoring it in the current election campaign. But four of the five admitted that they had never spoken to a congressman (or woman) about science. Richard Smalley of Rice University in Texas, who shared the chemistry prize for the discovery of fullerenes, said he hoped the prize would "give us more opportunity" to do so. □

NSF backs social sciences

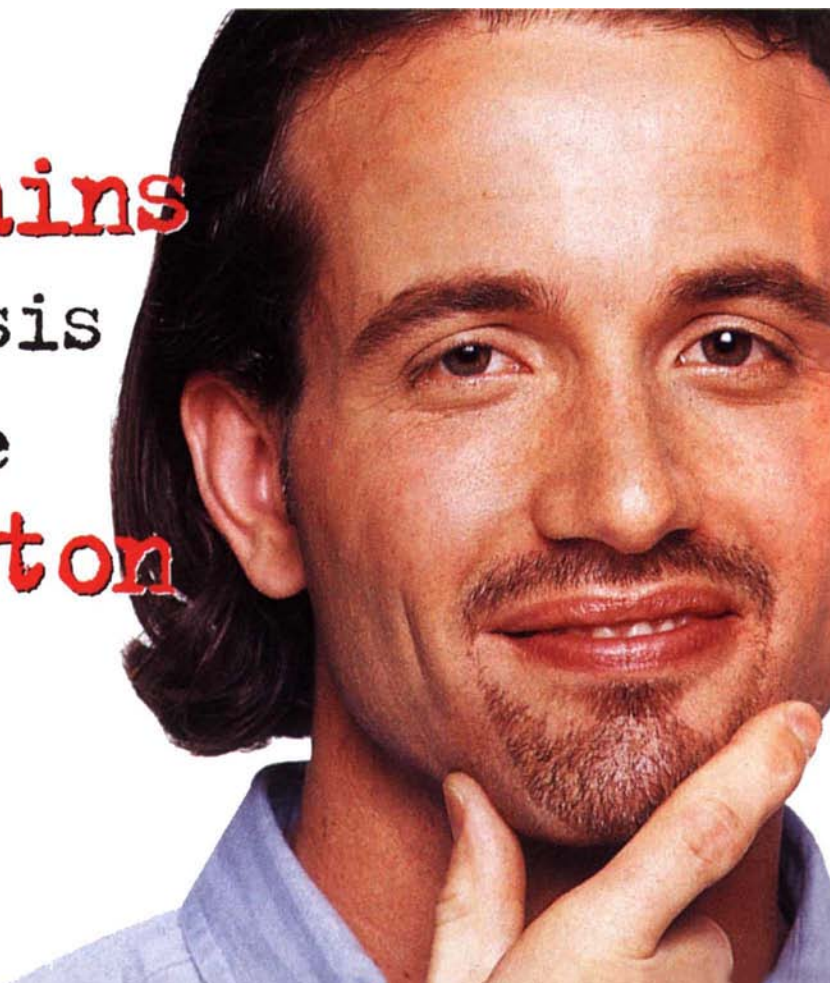
Washington. The National Science Foundation (NSF) has appointed Bennett Bertenthal as assistant director for its Directorate of Social, Behavioural and Economic Sciences. Bertenthal, a developmental psychologist at the University of Virginia, replaces Cora Marrett, who is returning to the University of Wisconsin.

The agency has come under intense pressure from Republicans in Congress to absorb the social science directorate's activities into the other six directorates, which fund "hard" scientific research. But Neal Lane, NSF's director, has repeatedly backed the directorate. □

Britain targets CFC smugglers

London. Britain's environment secretary John Gummer has promised to crack down on environmental crime, particularly the continued use of illegal ozone-damaging chemicals chlorofluorocarbons (CFCs). Launching a seminar on environmental crime in

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London last week, Gummer promised improved cooperation between the environment department, police, and customs to weed out such unlawful trade. The growing black market in CFCs is discouraging some refrigeration and airconditioning companies from using alternative chemicals, he said. □

Business bid bags bird name

London. An American businessman has had a new species of bird named after him for pledging the highest bid to save the bird from extinction. The *Vireo masteri* is named after Bernard Master, chairman of Health Power HMO in the United States. His donation to the charity Birdlife International, whose value has not been disclosed, will be used to fund conservation work in the Colombian forests of Rio Nambi, where the bird was discovered in 1991.

Newly discovered birds are traditionally named after their finders. But the *Vireo masteri*'s discoverers, Paul Salaman, a 19-year-old undergraduate student, and Gary Stiles, an ornithologist, auctioned the privilege to save the bird's habitat (see *Nature* 368, 781; 1994). □

Israel funds peptide research

Jerusalem. A consortium of Israeli drug and software companies and universities will receive US\$50 million over five years for the design and development of peptide drugs, the Ministry of Industry and Commerce has announced. Yoram Karmon, resident and chief executive officer of Peptor, a drug company, called the consortium "peptide heaven". According to Shuki Gleitman, the ministry's chief scientist, "there is a lot of academic knowledge and excellence in this field in Israel that we want to put on a business track".

Karmon agrees "there are many important research discoveries that have remained academic because the proper incentives have not been created". The current grant, he said, will create incentives for industry to translate academic research into practical results. □

Marie Curie revives grants

Brussels. Young scientists receiving training grants from the European Commission (EC) will in future be referred to as 'Marie Curie Fellows', Edith Cresson, the EC's commissioner for research, announced last week. The title will also be conferred on previous recipients of the funds, which are part of the EC's Training and Mobility of Researchers programme.

The object of the title-change, according to a statement from the commission, is to strengthen the "image and prestige" of the grants, and thus further improve employment prospects for their holders. Even now, 90 per cent of the 5,000 who have received a grant have remained active in their field after the grant's expiry. "The choice of Madame Curie's name underlines the quality of the fellows selected," says the commission.

A Marie Curie Association will also be created, whose goals will include increasing the visibility and prestige of the commission's training grants and helping to follow up past grant-holders. □

Corrections

In our issue of 3 October, a photograph of Enrico Garaci, president of the Italian National Research Council, was inadvertently used in place of that of Luciano Maiani (right), president of Italy's National Institute for Nuclear Physics, and recently appointed president of the council of the European Laboratory for Particle Physics (CERN) (see *Nature* 383, 370; 1996).

And David Jones, the author of the Daedalus column, and an early proponent of the possible existence of curved graphite molecules (see *Nature* 383, 561; 1996), is a chemist at the University of Newcastle upon Tyne, not the University of Nottingham as stated.

We apologize for both errors. □



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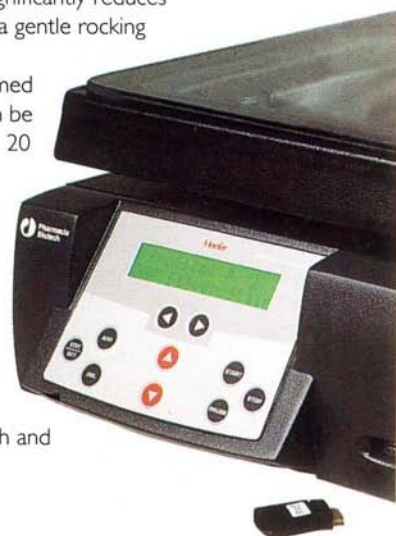
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What makes a good sport?

SIR — Professional sports, to survive, must attract fans, and so are under adaptive pressure to satisfy fans' desires by providing some combination of grace, strength, brilliance, violence and excitement. This is done, we suggest, with a finely calibrated mix of skill and chance in the outcome. Contests with too much chance are pointless as measures of relative ability. Those with too little chance in the mix provide no sus-

while those whose games are largely chance have long ones. Thus these sports, differing enormously in their particulars, converge towards the same reliability in a season. In contrast to what one might expect, season length is not an arbitrary product of historical, meteorological or other such constraints. Instead, almost all the variance in season length can be explained as an adaptation to fans' desires for the proper mix of

RELIABILITY AND SEASON LENGTH OF PROFESSIONAL LEAGUE SPORTS

Sport	Single-game reliability	Games per season	Season reliability
Baseball	0.008	162	0.566
Hockey	0.043	84	0.789
Soccer	0.065	42	0.703
Basketball	0.089	82	0.890
Football	0.118	16	0.681
Rugby Union	0.136	22	0.776
Rugby League	0.139	30	0.829

The split-half reliability coefficient, representing the extent to which a team's success in half the games predicts its success in the other half, can be adjusted (using the Spearman-Brown formula) to estimate the reliability of the entire season (twice as many games as the split half) or the reliability of a one-game season. This procedure was followed for 7 sports in 3 recent seasons. Season length is strongly negatively related to single-game reliability, $r(s) = -0.87$, $P < 0.01$. (For further details of methods, please contact the author.)

pense. The superior team should probably, but not certainly, win. Across all sorts of sports, those with the proper mix can thrive, while those that are too reliable or insufficiently reliable should never catch on. We find evidence that nine professional sports, from rugby to chess, control their reliability to create outcomes of roughly equal parts skill and chance. It is not that fans actually know how reliable a sport is, but rather their satisfaction depends on a sport having the right blend of skill and chance. Sports reflect this preference, shaped by the aggregated satisfaction of the public.

This notion of an optimal reliability for sports was tested by examining the extent to which sports regulate their reliability with the length of their seasons. Given a particular level of reliability within each game (which is influenced by spreading talent between teams, allowing small differences to have large consequences, rolling dice and the like), the reliability of the season is a function of the number of games played. A sport whose games are highly unreliable can increase the proportion of skill in the outcome by playing many games in a season. Similarly, a sport that, for structural reasons, has reliable games can increase the role of chance by having a short season. We calculated for seven league sports the reliability of each game, and compared this to the number of games played in a season (see table).

This demonstrated almost perfect compensation. The sports whose single games reliably assess talent have short seasons,

do fans of these sports have a level of reliability that they find most satisfying, but, in the aggregate, they must be exquisitely sensitive to the flow of information in sports to shape them so precisely.

If there is an optimal level of reliability, then it ought to appear not just in league sports, but in different sports with different structures. The predictability of two sports, tennis and chess, that depend on tournaments to assess skill is examined by measuring the extent to which players' rankings predict their success. While the nature of these sports and the structure of the contests are dramatically different from the league sports, the balance in the outcome between talent and chance is the same (details available from the author on request). This provides more support for the view that there is a level of predictability that is most satisfying, and that sports, regardless of the details, aim for this balance. As with natural selection, it need not be the case that sports deliberately strike this balance, but rather that those that do, succeed.

Nicholas Christenfeld

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Flaws in CTBT

SIR — Carrigan *et al.*¹ have recently shown how measurements of trace gas emissions on geological faults might be used as indicators of underground nuclear testing. Their experiment suggests that, of the 6.7×10^{15} becquerel (Bq) of xenon-133 produced by a one-kilotonne underground nuclear explosion, assuming that seepage through geological faults is similar to the conditions of the experiment, as much as 41 Bq m^{-3} could be present in the atmosphere, near the test site, after a few days.

This is within the sensitivity of existing techniques. A detector with a sensitivity of 20 Bq m^{-3} will be field tested next year². Such a detector would be appropriate in the mode proposed in the paper, namely in a well-timed challenge inspection on site. More sensitive detectors (in the mBq m^{-3} range) potentially exist³.

The text of the Comprehensive Test Ban Treaty (CTBT)⁴ speaks of equipping with xenon detectors some of the 80 or so fixed sites that will constitute the system of monitoring detectors designed to ensure compliance with the treaty. In this mode, detectors with a sensitivity in Bq m^{-3} , or even in mBq m^{-3} , would have a very high probability of yielding a false negative result — that is, of failing to detect tests of low yield (a few kilotonnes) if they do not take place in the direct vicinity of the detector.

Xenon detectors are interesting precisely for low-yield tests, for which other signatures are weak. But to be efficient, as ref. 1 clearly demonstrates, they should not be part of a fixed monitoring system, but should instead be used in on-site challenge inspections.

The experiment reported in ref. 1 thus shows that, in its present form, the CTBT does not have a verification architecture optimized for the best use of xenon detectors and ultimately to maximize the chance of successful verification.

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2. Perkins, R. & Casey, L. *Radio-xenons: Their Role in Monitoring a Comprehensive Test Ban Treaty* US DOE preprint: DOE/RL-96-51, June 1996.
3. Schell, W. *et al.* *Nucl. Instrument and Methods in Phys. Res. Sect. A* (in the press).
4. *Protocol of the Comprehensive Test Ban Treaty, Part 1: The International Monitoring System and Data Centre Functions*, Ch. C: Radio-nuclide monitoring, Para. 10 (UN Document A/50/1027, September 1996.).

Einstein for kg?

SIR — The kilogram is the only SI basic unit that is a multiple of another unit, the gram. I propose the adoption of a new name for the basic unit, which should stand on its own. I suggest einstein (symbol E).

Nobody will dispute the importance of Einstein's contributions to science in general, and to the discovery of the equivalence of mass and energy in particular. I believe he should be honoured by having an SI basic unit named after him. The gram has a long tradition and deep roots in science, so it should remain as $1 \text{ g} = 1 \text{ mE}$.

Doo Jung Jin

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Equipping researchers for the future

L. G. Georgiou and P. Halfpenny

The research equipment that scientists require to remain competitive is becoming more and more expensive. Unless new funding is found, existing allocation and management systems will have to change.

WHEN the competitive position of a country's science base is in question, arguments about the quantity and quality of research equipment soon come to the fore. This year, the first full-scale survey of research equipment in UK universities for almost a decade found that 79 per cent of departments were unable to perform critical experiments because of equipment shortages. Some £474 million (\$750 million) is needed to remedy the situation, but further capital cuts are scheduled over the next two years¹. In Japan, the arguments underpinning the proposed five-year plan for research and development (R&D) highlight the poor condition of university laboratories there, and 82 per cent of all R&D equipment in laboratories of the Science and Technology Agency and the Ministry of International Trade and Industry has passed its expected life². Yet enormous sums are being expended on equipment: for example, in 1994, US universities and colleges spent more than \$1.1 billion on equipment items costing more than \$20,000, as revealed in a joint survey by the National Science Foundation and the Scientific Research Society of America.

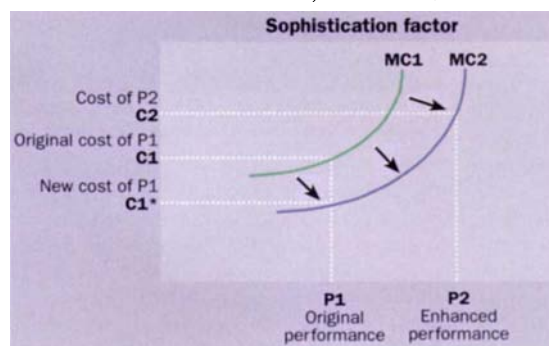
With equipment being crucial to scientific progress, surprisingly little attention has been focused on how best to fund and manage it. Several questions arise. Is the real cost of equipment needed to keep up with the field rising? Do grant-based funding systems provide adequately for equipment? Can funding constraints be mitigated by alternative management arrangements, such as concentrating equipment in the best laboratories, organizing sharing on a large scale or turning to industry?

Take first the question of cost. This is not simply a matter of inflation. Rather, the concern is whether the costs of research are rising faster than general inflation because of the demand for ever more sophisticated equipment. This problem was first raised in the 1960s when a pioneering study by UK government economists found substantial real-price growth rates per scientist for major items of equipment³. These were termed 'sophistication factors'.

Sophistication appears in several ways. Typically, classes of equipment follow trajectories of development that improve their research capability by increasing their performance (for example, their power, resolution or accuracy). At the same time, new 'families' of equipment types emerge that offer new research capabilities and only par-

tially replace equipment types already existing. These open up new opportunities for fields of science to become increasingly equipment-intensive (consider the automation of DNA sequencing in recent years).

One way to understand sophistication is to consider the relationship between price and performance. In a class of instruments, at any given time there is usually a range of prices for variants with different performance levels, and prices often rise faster than performance (that is, twice the power costs more than twice as much). Over time,



however, the real costs of equipment capable of a fixed level of performance are generally declining (often driven by the falling cost of electronic components). In this situation, for the sophistication factor to be positive, the cost of achieving improved performance must increase faster than any savings from the reduction in price per unit of performance. (You have probably paid around the same price for each of your last three personal computers, choosing vastly increased performance over vastly reduced price.)

The figure shows the marginal cost curve (MC1) for a performance characteristic. For a given application, a scientist requires a performance level of P1 which costs C1. Next, assume a technological advance that produces a new cost curve MC2. The original required performance level, P1, is now available at a reduced cost, C1*. But the field has also moved on and now requires an enhanced performance level, P2. If the cost of P2 on the new curve, C2, exceeds C1, then there is a net positive sophistication effect.

The question then is how the required performance level is set. On the demand side, the value placed by different research groups on a particular performance level will vary, giving a range of requirements. At any

given level, improved performance is redundant for some applications. The valuation is driven by the state of the subfield, the accuracy, say, of measurements required to support theories or test hypotheses in that area. Competition in science is likely to put upward pressure on the valuation of performance and hence its required level. At the limit are those groups that seek the highest available level and whose work therefore depends on 'state-of-the-art' equipment.

This framework allowed the measurement of the sophistication factor in the UK equipment surveys. Researchers in four fields (biosciences, chemistry, physics and mechanical, aeronautical and production engineering, defined as the UK funding council cost centres) were asked to provide four cost estimates for each instrument they used: original purchase price or construction cost (C1 on the graph, the actual cost when acquired, or the identifiable cost of construction); replacement cost (C1*, the estimated cost to purchase the equipment, or equipment of roughly equivalent function and capability at current prices); cost of an enhanced item (C2, the cost of equipment with capabilities that stand in similar relation to the current state of the field as the original item did at the time of its purchase); and cost of state-of-the-art equipment (the top of the MC2 curve, the estimated cost of the most sophisticated instrument in the field).

The ratios between these estimates provide the relevant measures of sophistication. The UK study gave the ratio replacement cost to original cost as 1.37, effectively a cumulative measure of equipment price inflation and of price reductions due to technical improvements. Approximating more closely to a measure of sophistication is the ratio between 'enhanced' and 'replacement' costs, 1.56. This incorporates the adjustment for advances in the expectations of those working in the field. The ratio state-of-the-art to replacement cost, at 2.67, indicates the premium required to remain at the forefront of research. This figure was highest for physics, where the cost of nuclear magnetic resonance machines rose rapidly across the four cost categories.

Although the precision of these results may be challenged, and the effect of entirely new types of equipment is excluded, results

over ten years have consistently shown positive sophistication factors. The policy implication is clear: equipment required to remain competitive in the field is becoming relatively more expensive, and, unless new funding is found, existing allocation and management systems will have to change.

Current systems for funding equipment broadly fall into three categories: block funding at the disposal of the laboratory, peer-reviewed grant funding and contract funding. Most researchers have access to some mixture of the three, but the nature of the mixture can have important implications for research equipment. Clearly block funding is the most flexible, but in the United Kingdom this is the category that has been most prone to cuts in recent years, only partially offset by increases in other sources. Grant funding against a research proposal may have been less subject to cuts, but it is normally tied to a proposed programme of work, and provision of equipment can be included only if it is specifically required to perform that work.

Two problems emerge from the predominance of this form of funding. First, with the current low success rates for proposals, planning becomes very difficult and whole programmes of work may be substantially delayed while the cycle of application and reapplication proceeds. In one case, new equipment worth \$600,000 was not usable because a funding application for the final component had been turned down. Second, there is a particular problem in obtaining funding for 'generic equipment': that is, equipment that is essential to the efficient operation of a laboratory but which has such wide application that it cannot be bought with a single grant.

The third form of funding, contracts from industry and others, offers no solution to the problem of generic equipment. Industry is even more likely to insist that the equipment it provides to universities is dedicated to the purposes of the projects it supports. An enquiry to research directors of leading UK companies clearly showed that they considered they were already making a substantial contribution to the academic infrastructure (8 per cent of the value of equipment purchases between 1991 and 1995) but that they believed that prime responsibility lies with the government. This opinion was backed up by the view that industry would relocate its collaborations in other countries if the infrastructure was not adequately maintained.

Case studies of research groups doing similar work in the United Kingdom, the United States and continental Europe showed varying situations. In general, the UK groups were more constrained by lack of equipment than their peers and had older equipment. Among the US groups, facilities rather than equipment seemed to be the main constraint, particularly a lack of space to accommodate equipment. Finding funds for servicing was also a problem. By con-

trast, in continental Europe the prevailing arrangement was centres of excellence provided with all the equipment they needed rather than having to assemble it piecemeal through a series of separate grants. One group told us that it had no equipment problems or needs. This apparently idyllic situation is not without its costs — national centres of excellence where resources have been concentrated are presumably funded at the expense of lesser groups.

IMAGE UNAVAILABLE FOR COPYRIGHT REASON REASONS

In physics, the cost of nuclear magnetic resonance machines rises especially rapidly.

What of management solutions? Does the answer lie in moving funds from weaker performers to ensure that the stronger are properly equipped? The problem is that substantial concentration already exists. In the United Kingdom, two institutions accounted for 16 per cent of equipment expenditure in the past five years, five for 27 per cent and 13 for 50 per cent. If all equipment funding was taken away from the bottom half of universities in the spending league, this would pay for only a 10 per cent increase for the top half. A similar situation prevails in the United States, where the top 20 institutions account for about a third of expenditure and the top 40 for about a half. Nonetheless, in the United Kingdom at least, further concentration is on the agenda as the review conducted by Sir Ron Dearing considers the future of higher education (see *Nature* 382, 381; 1996), with support for this strategy also coming from industry.

With or without concentration, pressure is bound to increase for better use to be made of existing equipment, which means more sharing. At first sight the opportunities are good, with the UK survey reporting that more than a third of equipment had spare capacity for additional use by researchers from outside the department. But equipment with spare capacity tends to have poorer technical capabilities and to be older (two-thirds had been purchased before 1991). There are examples around the world of successful equipment-sharing schemes, ranging from databases of resources, through equipment pools, to large facilities with one or more items available for external use. But these account for only a small

proportion of equipment, and for good reasons. An earlier study revealed that sharing requires substantial management input and is far from cost-free¹. Charging and access arrangements need to be established and a higher level of maintenance budgeted for. Supervision is necessary to prevent damage being inflicted by inexperienced users or, alternatively, staff are needed to provide a service for users. In addition, psychological barriers have to be overcome. They are particularly high when existing equipment is involved to which proprietorial attachments have already been made. Good research is unlikely to be performed under the baleful eye of an aggrieved enforced 'host'. Unless the sharers are part of a group with some loyalty to each other, it is likely that the equipment will be used much as a hire car is driven rather than with the care taken when borrowing a friend's vehicle.

What then is the way forward? There are no easy answers. Present deficiencies and the pressure of sophistication clearly mean that more funding is needed for research equipment, together with greater flexibility in the ways in which it is disbursed. Although the input from sources such as industry and charities may increase, the base is too low for the whole answer to lie there. Governments cannot therefore abnegate their responsibilities, particularly in the face of international competition, for attracting and retaining industrial R&D investment. Greater concentration seems inevitable, but unless many scientists are to be cut off from experimental work this will have to be accompanied by a substantial increase in the access offered to researchers from outside the privileged institutions. Geographical constraints imply regional centres. With the management costs involved in sharing, only large facilities would offer sufficient economies of scale, and to overcome psychological barriers new investment will be needed rather than reallocation of existing equipment. In the final analysis, scientists will have to face the same choices as those being made in management and service industries across the world. To stay competitive, the ratio between capital and labour needs to change. □

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Knitting a finer net for photons

E. Yablonovitch and D. F. Sievenpiper

By etching an array of tiny holes in a semiconductor crystal, one can prevent light of certain wavelengths from propagating. Now these photonic bandgaps are approaching the technologically fertile optical regime.

SURPRISINGLY, science has never found a way to control optical waves in all three directions of space. Electromagnetic waves are like gelatine — squeeze in one direction and they leak out a different way. Until recently, we could control only one direction at a time, as in the face-to-face mirrors of laser cavities. But squeezing the gelatine in all three directions at once was beyond our imagination, and we needed a lesson from nature.

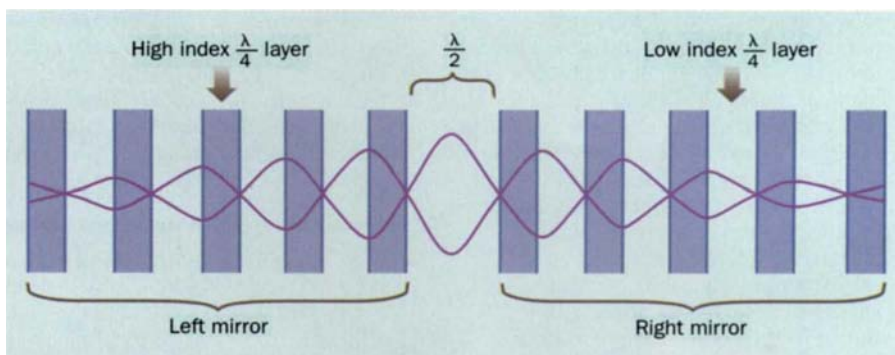
On page 699 of this issue¹, Krauss, De La Rue and Brand present the latest development in the field of photonic-bandgap structures, or photonic crystals. Photonic crystals are engineered three-dimensional structures that are an attempt by scientists to control electromagnetic radiation, to bottle it up and to trap it in the tiniest possible volume, or indeed to prevent atoms from emitting light in the first place. Such exquisite control would mean that we could make lasers much smaller and more efficient than the ones we have now — a step in the ongoing miniaturization revolution of both electronics and optoelectronics.

The lesson was that nature had already solved the problem, not for electromagnetic waves, but for electron waves in natural semiconductor crystals. Semiconductors possess the celebrated 'forbidden bandgap', the basis for the electronics, information and communications revolution. Try as it might, an electron wave of the wrong energy in a natural semiconductor-crystal structure can find every possible propagation direction forbidden, blocked by diffraction off one plane of atoms or another.

So the challenge for us is to make an artificial three-dimensional dielectric structure, but for electromagnetic waves rather than electron waves. This has led to an epic search for artificial crystal structures that would have such a 'photonic bandgap' or PBG. After many false starts, experimental and computational trials, and premature declarations of success, this search ended a few years ago^{2,3}.

attenuation of a factor of 100 for light in the forbidden wavelength range). Although their structure has a gap for only one component of polarization (with the magnetic field parallel to the pillars of air), these results are an important step towards complete photonic-bandgap structures at optical wavelengths.

Several other groups have also produced short-wavelength photonic crystals,



A periodic stack of plates can confine light, but in only one dimension, leaving it free to leak out at the sides. Structures with two-dimensional periodicity can be devised to confine light to a line, and three-dimensional periodicity can forbid propagation in any direction.

Crystal geometries are now known that have a photonic bandgap, both in two and three dimensions, and which have been tested using microwaves in large-scale models.

This has led to world-wide competition to make these tiny structures at the scale of optical wavelengths, where the communications and optoelectronics industries work. Although the ultimate aim is three-dimensional structures, most researchers around the world have opted to first address the easier problem of making two-dimensional photonic crystals.

The paper by Krauss and colleagues¹ shows that they currently lead in the race to produce two-dimensional PBG structures at optical wavelengths. Using a combination of electron-beam lithography and plasma etching, they punched a honeycomb lattice of 100-nm holes in a crystal of AlGaAs, to produce a crystal with a bandgap in the 800–900-nm range — the shortest-wavelength gap to date in a semiconductor material. They have also measured the transmission characteristics of their crystal using a waveguide arrangement, demonstrating an impressive bandgap rejection of up to 20 dB (that is, an

but much of the work has been done in lead-oxide glass, drawn out into fibres. Various structures have been made in this way, one with a near-infrared gap⁴, and others with gaps spanning the visible range⁵ down to 350 nm. Unfortunately, lead glass has a refractive index of only 1.65, which is not enough to produce a simultaneous gap for both electromagnetic polarizations. For the favourable geometry of a triangular lattice of air-filled rod-shaped holes embedded in a dielectric, a polarization-independent gap requires a minimum refractive index⁶ of 2.66, which is easily achieved in semiconductors, but out of reach of most glasses. In two-dimensional optical fibres, however, it may be possible to use photonic-crystal periodicity instead of refractive-index guiding to confine the light⁷.

Porous silicon is also an impressive photonic-crystal material, the first in which a polarization-independent, two-dimensional gap has been achieved⁸, albeit centred at the infrared wavelength 4.9 μm. The inverse structure — a lattice of silicon pillars — has also been studied, but a bandgap has not been achieved⁹.

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Some III–V semiconductor photonic crystals have likewise been made^{10,11}, but their transmission properties have not been measured.

As optoelectronic technology becomes ever finer, we will be able to make tiny lasers half a wavelength on a side. They may operate on new principles, like the single-mode light-emitting diode that would emit spontaneously but have many of the advantages of lasers as well. It would be reliable and insensitive to tem-

perature, and would not require a threshold current. At the opposite extreme, macroscopic photonic crystals show much promise for microwave communications.

Krauss and colleagues have made important progress by synthesizing and testing a semiconductor-based two-dimensional photonic crystal with a gap centred at a record short wavelength. By concentrating on the semiconductor alloy AlGaAs, they have the necessary refractive index to eventually produce a gap for

all electromagnetic polarizations. This race is far from over, and we will undoubtedly soon hear of more progress from other groups. Furthermore, the challenge of creating fully three-dimensional photonic crystal nanostructures has hardly begun. □

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SPONGIFORM ENCEPHALOPATHIES

A suspicious signature

Adriano Aguzzi and Charles Weissmann

THE infectious agent that causes transmissible spongiform encephalopathies — the prion — is the subject of one of the most passionate controversies of contemporary biology. The questions regarding the nature of this agent touch on a grave issue of public health: the possible link between bovine spongiform encephalopathy (BSE) and vCJD, a new variant of Creutzfeldt–Jakob disease (CJD) which has mainly affected young people in the United Kingdom (see table)¹. Such a link has been shown to be plausible because a variety of animals can acquire BSE through the oral administration of brain tissue from BSE-infected cattle. Now on page 685 of this issue², John Collinge and co-workers describe an exciting new approach for tracing the passage of individual prion strains within and between species, and they suggest that vCJD resembles BSE rather than acquired or sporadic CJD.

Transmissible spongiform encephalopathies are spread by an agent which, unlike any other known agent of disease, apparently lacks informational nucleic acids³. Along with an impressive body of genetic evidence^{4–6}, this observation led to the development of the ‘protein only’ hypothesis — that the prion is simply the modified form of a normal protein named PrP^C, which is encoded by a single-copy host gene⁷. The surmised infectious form of PrP has been designated PrP^{*} (ref. 8), and an abnormal form of PrP^C, called PrP^{Sc} (or PrP^{res}, for protease-resistant), has been equated with PrP^{*}. PrP^{Sc} accumulates during the advanced stages of most cases of transmissible spongiform encephalopathies, and it is characterized by its partial resistance to proteolytic digestion and by its tendency to aggregate.

PrP^C and PrP^{Sc} seem to be chemically identical, so they may represent conformational isomers. PrP^{Sc} is thought to

propagate by interacting with PrP^C and causing its conversion into PrP^{Sc}. As predicted by the ‘protein only’ hypothesis, PrP^C is essential for the propagation of infectivity⁹, for clinical disease⁹ and for brain pathology¹⁰. But this hypothesis cannot account for the occurrence of distinct prion strains that can be propagated indefinitely in hosts that are homozygous

suggested. First, PrP^{*} or PrP^{Sc} could be associated with an accessory molecule, such as a small nucleic acid¹², which modulates the phenotype of the prion without being essential for infectivity. However, there is no evidence for a nucleic acid of this kind. Second, PrP^{*} could undergo secondary modifications, such as glycosylation. Certain polysaccharide residues could then specifically target the molecule to cells which in turn glycosylate PrP in an identical fashion. This is known as the ‘target-cell hypothesis’ (K. H. Meyer, personal communication). Finally, PrP^{*} or PrP^{Sc} could exist in various different conformations (at least one for each prion strain), with each type of prion being capable of imparting its own conformation to the PrP^C molecule with which it interacts. More than one protein chemist has declared this idea to be insane — and yet this is precisely what is implied by a growing number of studies.

Western blot analysis of PrP from extracts of normal or prion-infected brains reveals three major bands, corresponding to PrP that has two, one or no polysaccharide chains attached to the amino terminus. In normal brain extracts, treatment with proteinase K degrades all of the PrP; however, in prion-infected brain, protease treatment leads to increased mobility of the three protein species because of amino-terminal truncations. Presumably, the

changed conformation of PrP^{Sc} protects a large part of the molecule against degradation. Bessen and Marsh¹³ showed that when two strains of PrP derived from mink were propagated in inbred Syrian hamsters, they gave rise to PrP^{Sc} molecules that had distinct and heritable migration patterns following proteinase K treatment. This difference in mobility was shown to be due to cleavage at distinct sites.

The most common form of prion disease in humans, Creutzfeldt–Jakob disease, occurs in four forms: a familial form, linked to mutations in the PrP gene; an iatrogenic or acquired form, due to inad-

Differences between sporadic and variant CJD

	Sporadic CJD	New variant of CJD (vCJD)
Typical age of onset	55–70 yr	19–39 (median 28) yr
Presenting features	Dementia, myoclonus	Behavioural changes, ataxia, dysaesthesias
Clinical course	Rapidly progressive	Insidious onset, prolonged course
PRNP genotype (codon 129)	Predominantly homozygous	Met/Met homozygosity in 100% so far
PrP ^{Sc} deposits	Synaptic deposits, rarely plaques	Prominent florid plaques
PrP ^{Sc} banding pattern	Type 1 and type 2*	Type 4 (similar to experimental BSE in mice, macaques and other species)

* Type 3 in iatrogenic cases with intramuscular inoculation

with regard to their PrP genes. Prion strains are identified by the disease incubation time or by the pattern of brain lesions¹¹. Stable prion strains could be easily explained if the infectious agent were virus-like: strains would then be encoded by the viral genome. However, if the prion is a conformational isoform of PrP^C, then all of its phenotypic characteristics should be determined by the host PrP genes alone and any prion strain should, after a few passages in hosts of the same species, become monomorphic.

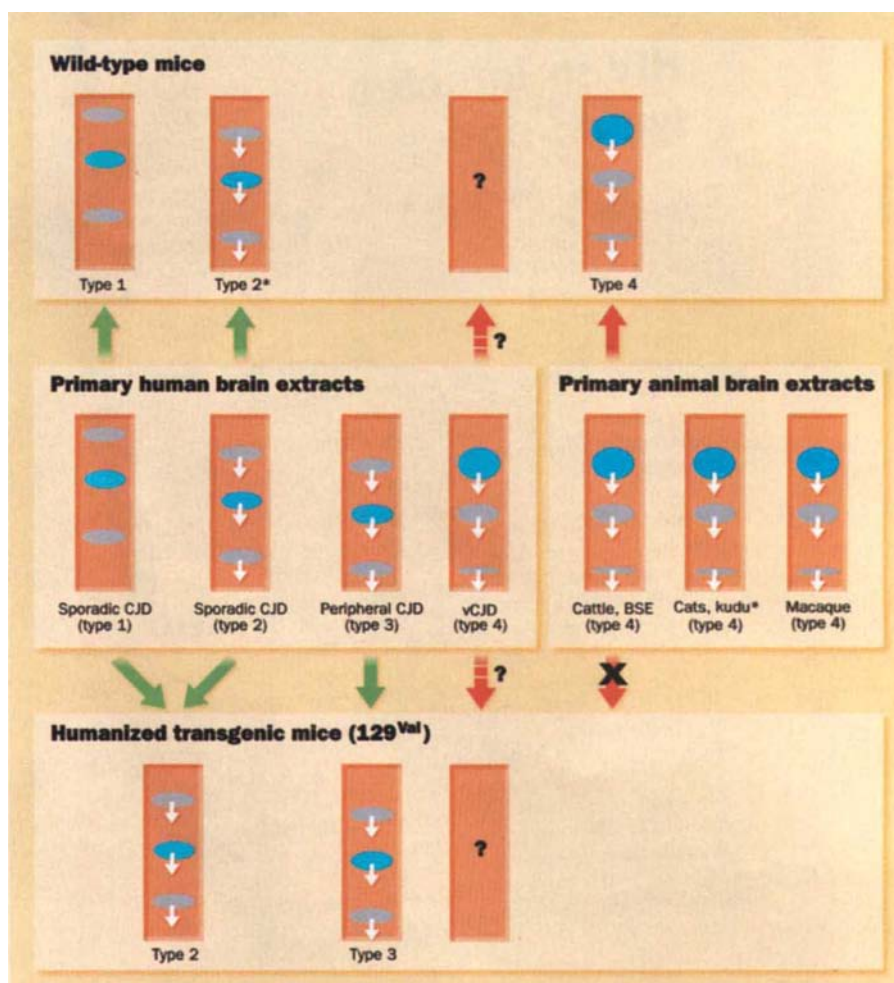
How can the existence of different strains be explained by the ‘protein only’ hypothesis? Three possibilities have been

vertent exposure to CJD-contaminated material, notably human cadaveric growth hormone; variant CJD, which is attributed to ingestion of BSE-contaminated bovine products; and sporadic CJD — the most common form — which is of unknown aetiology.

Parchi *et al.*¹⁴ demonstrated that protease-treated PrP^{Sc} in sporadic CJD has two different mobility patterns which, in conjunction with a methionine/valine polymorphism at codon 129, specify four distinct clinical entities. Collinge and colleagues² have now extended these observations to iatrogenic and vCJD. As well as the mobility of protease-treated PrP^{Sc}, they investigated the relative intensities of the three bands that reflect the glycosylation state, and on the basis of this they defined four 'types' of pattern (see figure). Protease-digested PrP^{Sc} from cases of sporadic CJD gave rise to type-1 or type-2 patterns, and PrP^{Sc} from iatrogenic cases gave rise mainly to the type-3 pattern. Astonishingly, all vCJD samples were of the type-4 pattern, which was not seen in any other form of CJD. Moreover, extracts from the brains of BSE-infected cattle, cats or kudu that were thought to have acquired BSE, and macaques that were infected experimentally with BSE, all showed a type-4 pattern. This pattern is mainly characterized by a high proportion of diglycosylated PrP^{Sc}.

Experiments were also done to transmit the different forms of human spongiform encephalopathies to wild-type mice or to mice that were transgenic for the human PrP genes. Sporadic or iatrogenic CJDs with patterns of type 1, 2 or 3 gave rise to type-2 or type-3 patterns in mice carrying human transgenes. Brain extracts from BSE-infected cattle gave rise to a type-4 pattern in wild-type mice, again with a characteristically high proportion of diglycosylated species. So the supposed conformational strain specificity is conserved after transfer between species. Notably, the patterns given by vCJD after transmission to the mouse have not yet been reported, but they are awaited with interest.

How do the different patterns come about? Protease-treated PrP^{Sc} species are thought to have different mobilities



On the western blots done by Collinge and co-workers², different prion strains show characteristic patterns of PrP^{Sc} glycoforms, which are inherited even when the infectious agent is passed between different species. Green arrows indicate successful transmission. The major glycoform of PrP is drawn in blue and the arrows on the western blots indicate increased mobility compared with type 1. Provocatively, the vCJD pattern is similar to that seen in cows and other species with BSE. *J. Collinge, personal communication.

because of differing protein conformations. This is also true for the bands corresponding to non-glycosylated PrP, so the variability cannot be ascribed to different polysaccharide residues. A closer inspection of the western blots suggests that even within a single pattern-type the species have variable mobility, so there may be even more different conformations represented among the samples. The unique feature of the type-4 pattern — namely the high ratio of diglycosylated to unglycosylated PrP — may reflect increased susceptibility of the diglycosylated form to BSE-mediated conformational change. Alternatively, cells that preferentially produce the diglycosylated form may be more readily targeted by the BSE agent.

The findings of Collinge and co-workers² support the proposal that strain specificity is linked to distinct, heritable conformational states, and they may help in the classification of human and animal prion diseases. Moreover, they provide further evidence that the BSE agent has

been transmitted to man. This conclusion will be further reinforced if vCJD and BSE impart a similar signature to PrP^{Sc} when transmitted to mice. One obvious extension of this work will be to compare the PrP^{Sc} pattern of natural sheep scrapie with that of sheep infected with experimental BSE. A pattern difference, if present, may be used to determine whether, and to what extent, BSE has been transmitted to sheep by contaminated feed — a question of particular importance for public health. Also interesting will be the search for type-4 PrP^{Sc} in elderly patients with encephalopathy, as the aged have thus far, and for no obvious reasons, been spared by vCJD. □

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The rudists re-examined

David Jablonski

PERHAPS the most spectacular departure from the mollusc body plan in the 500-million-year history of the phylum occurs in the rudist bivalves, an extinct group of marine molluscs as fascinating and bizarre as their contemporaries, the dinosaurs. The rudists present an intriguing array of functional, ecological and evolutionary puzzles; fresh perspectives were presented on many of these last month, when most of the world's experts convened in Marseilles*.

Rudists (the popular term for members of the superfamily Hippuritacea; from the Latin *rudis*, rough) abandoned the usual bivalve form, in which the paired shells or valves are essentially mirror images of one another as in scallops and cockles, to generate a conical, barrel-shaped or corkscrew-shaped lower valve and a cap-like upper valve held in place by an elaborate tooth-and-socket system; some rudist species were small and delicate, but lengths exceeding 1 m and diameters exceeding 60 cm were not uncommon. Even when they re-evolved a more symmetrical shell form, they did so in outrageous fashion, as in the Caribbean form *Titanosarcotites* (no. 11 in the figure), which is more than 2 m long and resembles "two giant, coil-toed Persian slippers placed heel-to-heel"¹.

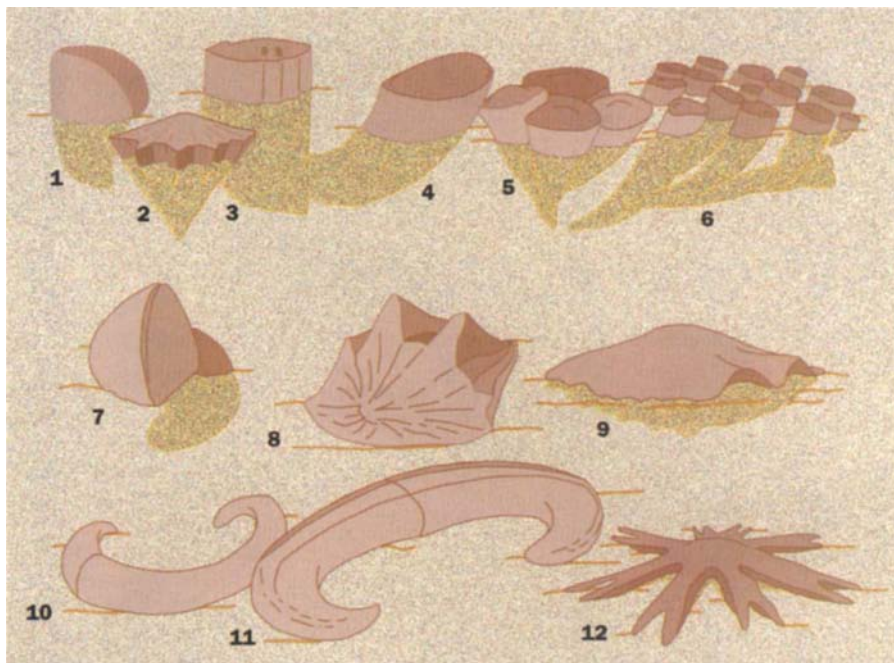
Rudists have often been described as constructing reefs and as having out-competed the corals that today thrive in clear tropical waters^{2,3}. They certainly were overwhelmingly concentrated in shallow tropical seas from their first appearance in the Late Jurassic (about 155 million years ago) to their extinction along with the dinosaurs, ammonites and many other groups at or near the end of the Cretaceous (65 Myr ago). Further, whereas some rudist species were sparsely distributed across the entire continental shelf, others occurred in extremely dense populations within a narrow, shallow zone, forming shelly limestone bodies several metres thick and many square kilometres in extent. In fact, the enormous production of rudist limestones in the Cretaceous tropics appears to be a carbon sink so massive that no attempt to understand the carbon cycle of the Cretaceous 'greenhouse world' can afford to discount it (P. W. Skelton, Open Univ., Milton Keynes; J.-P. Masse & J. Philip, Univ. Provence, Marseilles).

Those limestone bodies are now host to many of the huge petroleum reserves of the Middle East and the Gulf of Mexico, lending rudists an economic cachet as oil

companies reconstruct the geometries of dense rudist formations in order to predict profitable drilling sites.

A few rudist formations do show some of the hallmarks of a reef — a cohesive, solid structure with some topographic expression above the sea floor¹⁻³ (S. Götz & B. Höfling, Univ. Munich; D. Schu-

man, Tech. Univ. Darmstadt) — but they seem to be exceptions and a close analogy with today's reef-building corals appears to be unravelling^{4,5}.



In the Cretaceous Period, rudist bivalves evolved a remarkable diversity of shell morphologies, from conical forms partly embedded in the sediment (1–6), to corkscrew-like and flattened forms (7–9), and arc-shaped and stellate creatures that reclined on the sediment (10–12). Lengths range from 15 cm (1) to 2 m (11). (From ref. 4.)

mann, Tech. Univ. Darmstadt) — but they seem to be exceptions and a close analogy with today's reef-building corals appears to be unravelling^{4,5}. Rudists are emerging as a unique evolutionary experiment in tropical seas, exploiting habitats and lifestyles that correspond only loosely, if at all, to modern reef structures. Far from producing a framework, many rudists were solitary, or formed meadows of small clusters that floated in a muddy sea floor. Flume experiments show that partially embedded shells, if inclined downstream of prevailing currents, could make use of detritus brought up from the sediment surface by eddies in the lee of the animal — a feeding strategy rare or absent in modern corals (E. Gili, Univ. Autònoma, Barcelona; M. LaBarbera, Univ. Chicago). For many species, a better modern analogue might be the Great Pearl Bank in the southern Arabian Gulf, with its dense populations of the fan mussel *Pinna* (G. W. Hughes, Saudi Aramco, Dhahran), or perhaps associations of the giant clam *Tridacna* found on Indo-Pacific

reef flats and in back-reef lagoons. Given their differences, did the rudists really out-compete and marginalize contemporary corals? We now realize that corals were diverse and abundant in the Cretaceous tropics, and in many settings even grew on and around rudists^{5,6} (J.-P. Masse, Univ. Provence, Marseilles; P. Garcia-Barrera, G. Alencaster, J. Aven- daño-Gil & L. Omaña, Univ. Nacional de Mexico; D. Schumann, Technische Univ., Darmstadt). Dense, monospecific rudist populations may have lacked corals,

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*Fourth International Conference on Rudists, Marseilles, France, 9–16 September 1996.

and their release after the rudists became extinct, now appears an oversimplification.

A more stubborn question is whether the rudists had endosymbiotic algae, such as the zooxanthellae seen in many corals and in *Tridacna*. Such a partnership has been suggested, but not proven, for those genera that have massive shells, great abundance, a preference for shallow depths, or shell margins that could have exposed tissue-bearing photosynthesizing symbionts^{2,3,6,7}. But the presence of rudists in settings that must have been bathed in turbid waters, and the absence in most genera of clear-cut morphological adaptations for tissue exposure⁶, suggests that zooxanthellae were not present in all rudists — perhaps not surprisingly, given the diversity of the group and the uneven

distribution of symbionts among other bivalves and even among corals.

New work on oxygen and carbon isotopes may help to settle the question: rudist growth rates, estimated from what appear to be annual temperature cycles in a few well-preserved shells, can be as high as 50 mm yr⁻¹, implying calcium carbonate production rates up to 20 kg m⁻² yr⁻¹ — values that fall within the range for modern symbiont-assisted coral reefs⁸ (T. Steuber, Univ. Köln). These isotopes occur in ratios far from oceanic equilibrium, and in patterns that resemble those seen in corals, but even such pronounced metabolic fractionation cannot be taken as a sure sign of symbiont activity.

Finally, the rudists suffered a number of extinction events, in the mid-Aptian (about 117 Myr ago), at the end of the

Cenomanian (about 94 Myr ago), but each time re-diversified well beyond pre-extinction levels. Indeed, the rudists were at their most diverse just a few million years before their disappearance, the exact timing and cause of which remain hotly debated.

The rudists still present many enigmas, but the new burst of research, ranging from painstaking field and laboratory work in the classical mode to novel approaches in isotopic biogeochemistry and experimental biomechanics, has given us a deeper understanding, and defined the next set of questions to be tackled. □

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NEUROBIOLOGY

Memories of nicotine

Daniel S. McGehee and Lorna W. Role

THE first discussions linking the enhancement of memory with the use of tobacco almost certainly occurred long before tobacco was introduced to Western society. In 1659, Dr Giles Everard wrote:

“...to strengthen the memory the fmoke is excellent taken by the noftrils, for it is properly belonging to the brain, and it is eafily conveyed into the cels of it and it cleafeth that from all filth (for the brain is the Metropolis of flegme, as Hippocrates teacheth us...)” From *Panacea; or The Universal Medicine, Being a Discovery of the Wonderfull Vertues of Tobacco Taken in a Pipe, with its Operation and Use both in Physick and Chyrurgery*. (Fig. 1)

Although nearly all of the “wonderfull vertues of smoke” have now been disproved, the theory that nicotine enhances some forms of memory has remained¹. The

underlying mechanisms are still unknown, but on page 713 of this issue Gray *et al.*² provide evidence that nicotine increases the strength of synaptic communication between neurons in the hippocampus — a centre for learning and memory.

Many groups have examined the cellular events that control the strength of connections (synapses) between nerve cells in the hippocampus³, and these studies have provided insight into the basic processes that are thought to underlie learning and memory. Hippocampal neurons express several classes of nicotinic acetylcholine receptors (nAChRs), and there is a differential subcellular distribution of the distinct nAChR subclasses⁴. Furthermore, nicotinic cholinergic systems are believed to be important in memory — nicotine has a memory-enhancing effect, and there is a loss of cholinergic innervation from the basal forebrain in pathologies that affect memory, such as Alzheimer's disease.

Gray *et al.*² now show that at the concentrations of nicotine detected in arterial blood during smoking, there is enhanced glutamate-mediated transmission of nerve impulses in hippocampal cultures. They also show that nicotine increases the levels of intracellular calcium measured directly from presynaptic terminals in hippocampal slices, providing the best evidence to date that the nAChRs that mediate enhanced neuro-

transmitter release are localized to these presynaptic terminals. Such high-resolution analysis constitutes a significant advance in our understanding of the potent effects of nicotine at an important site in the brain. The mechanism by which nAChRs are thought to be involved in synaptic transmission at glutamatergic synapses is outlined in Fig. 2.

The paucity of evidence for the direct involvement of nAChRs in mediating synaptic transmission in the central nervous system is epitomized by the class of neuronal nAChRs that are blocked by α -bungarotoxin (α -BGT). Anatomical studies revealed widespread binding of α -BGT in the brain; however, the lack of evidence for α -BGT-sensitive, nicotine-gated currents branded the α -BGT-binding sites as ‘nonfunctional’ for decades⁵. However, the α -BGT-binding sites were resurrected by the demonstration that three of the nAChR α -subunits — $\alpha 7$, $\alpha 8$ and $\alpha 9$ —

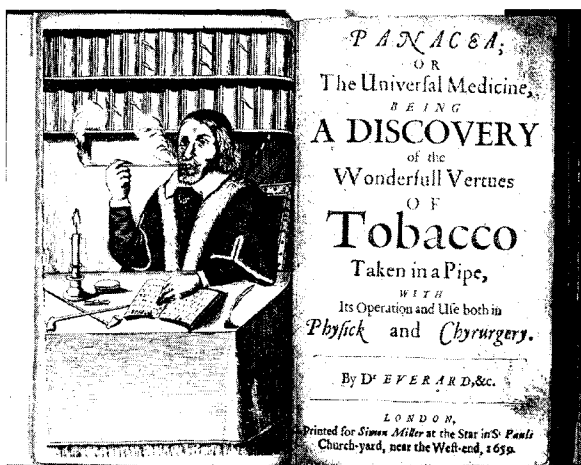


FIG. 1 As well as espousing numerous “vertues of tobacco”, Dr Everard points out that abuses of “... this noble herbe ... are a great force to shorten your daies”.

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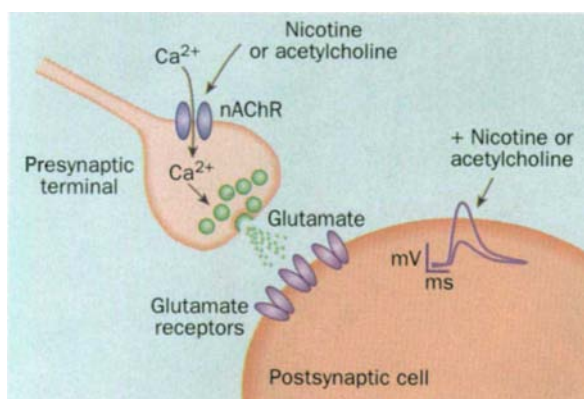


FIG. 2 Low concentrations of nicotine activate nAChRs expressed on presynaptic terminals, and this induces an influx of Ca²⁺. The subsequent increase in intracellular Ca²⁺ enhances vesicle fusion and exocytosis. At a synapse (such as the one illustrated here), nicotine enhances vesicular release of the excitatory neurotransmitter glutamate. The inset depicts an increase in the magnitude of the evoked postsynaptic voltage response due to the increased release of glutamate from the presynaptic terminal in the presence of nicotine or acetylcholine¹². Nicotine-induced Ca²⁺ influx also enhances the spontaneous release of glutamate, as shown by Gray *et al.*².

could form homomeric channels in *Xenopus* oocytes, and that these were blocked by nanomolar concentrations of α -BGT⁶⁻¹⁰.

Nicotinic nAChRs that include the $\alpha 7$ subunit are highly permeable to calcium^{8,11}, indicating that these receptors are ideally suited to induce calcium-sensitive processes such as neurotransmitter release. Although the enhancement of excitatory synaptic transmission by α -BGT-sensitive nAChRs has been observed in other systems^{12,13}, the involvement of $\alpha 7$ in mediating the presynaptic effects of

nicotine seems to be the exception rather than the rule: it is well established that the activation of α -BGT-insensitive nAChRs (for example, $\alpha 4\beta 2$) enhances the release of numerous neurotransmitters in several regions of the central nervous system^{11,14,15}. Furthermore, these α/β -type nAChRs also have significant calcium permeability¹⁶.

Although nicotine can enhance neurotransmitter release in many regions of the central nervous system, it is likely that the activation of presynaptic nAChRs is just one of many steps required for nicotine to alter behaviour or cognition¹⁷. The cascade of events that follows an initial activation of presynaptic

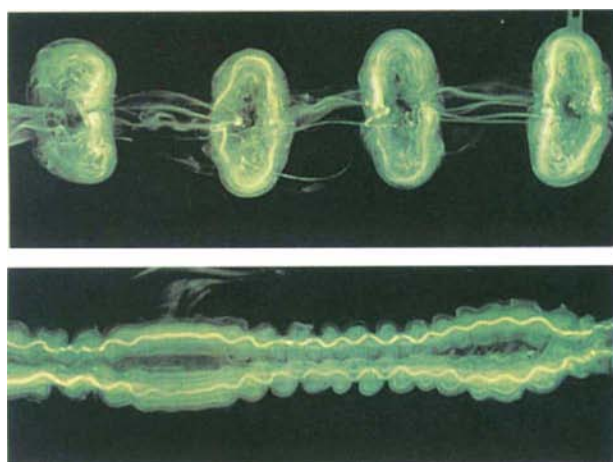
nAChRs most notably includes the entry of calcium as well as the desensitization and, ultimately, inactivation of the nAChRs. Furthermore, all of the nAChR-mediated effects depend on the total concentration of the various agonists. Thus, the level of endogenous acetylcholine upon which the exogenously administered nicotine is superimposed will contribute to the net physiological effect.

Even the first cigarette of the day — anecdotally renowned for being the best — may deliver nicotine to presynaptic

nAChRs that have already been exposed to endogenous neurotransmitter. In this context, will the exogenous drug further activate the nAChRs and enhance neurotransmitter release as suggested by *in vitro* analyses? Or is the additional nicotine simply 'excess' agonist which is sufficient to drive presynaptic nAChRs into the desensitized, non-conducting states? In the latter case, exogenous nicotine would decrease transmission at sites where endogenous acetylcholine release enhances synaptic gain. Furthermore, the effect of nicotine at a given synapse will depend on the acetylcholine-mediated activation and inactivation of the specific nAChRs that are expressed (α/β type or $\alpha 7$ -type, or both). In the absence of information on the levels and possible sources of acetylcholine, the relative contributions of activation and inactivation of presynaptic nAChRs to the effects of nicotine in the central nervous system is still beyond reach. Nevertheless, the definitive demonstration by Gray *et al.*² that nicotine potentially enhances transmission in the hippocampus is unquestionably an important step towards recalling nicotine as a powerful modulator of memory. □

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Clever use of light and unparallelled flow



THESE striking images are from this year's *Gallery of Fluid Motion* (*Phys. Fluids* 8, S1-S12; 1996).

On the left, a fluorescent dye traces the long- and short-wavelength instabilities of a pair of counter-rotating vortices. In the upper panel, the two initially straight, parallel vortices have broken up and reconnected to form rings — an effect that has been predicted but never produced before (T. Leweke & C. H. K. Williamson, Cornell Univ.).



On the right, a soap film is suspended between two vertical nylon wires, and flows at a terminal velocity of two or three m s⁻¹ down past an array of comb teeth and a knife (far right), whose sharper edges produce particularly fine structure that may be useful in generating turbulence. Interference fringes show up the thickness variations in the vortices (M. A. Rutgers, X.-L. Wu & W. I. Goldburg, Univ. Pittsburgh).

Stephen Battersby

Notch, stroke and dementia

Thomas Gridley

THE Notch intercellular signalling pathway is essential for proper embryonic development in organisms as diverse as insects, nematodes and mammals (reviewed in ref. 1). So it is somewhat surprising that few associations have been described between components of this pathway and human disease. Previously, the only evidence connecting Notch signalling and disease was the involvement of *Notch1* gene translocations in a minority of T-cell lymphoblastic leukaemias². But the paper by Tournier-Lasserre and colleagues on page 707 of this issue³ now provides important new data linking the Notch signalling pathway to familial causes of stroke in humans.

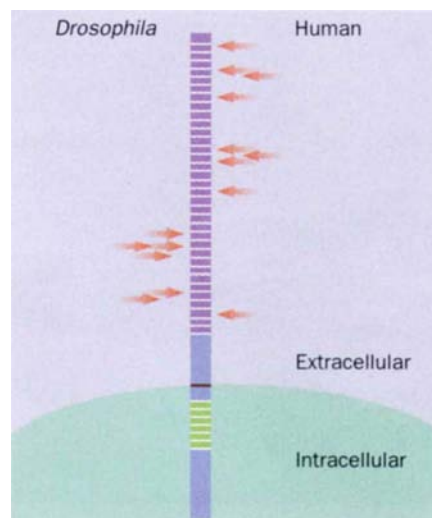
In developed countries, stroke is the third leading cause of death and the primary cause of acquired physical or cognitive impairment. Recurrent strokes are a feature of a recently described familial syndrome known as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy). Affected individuals exhibit a variety of symptoms, including recurrent subcortical ischaemic strokes, progressive vascular dementia, craniofacial paralysis, migraine, and mood disorders with severe depression⁴. These symptoms usually start to appear at about 45 years of age, and patients typically die by 65. The condition is believed to be largely undiagnosed and, as such, the prevalence is not precisely known.

In previous work, Tournier-Lasserre and colleagues mapped the CADASIL gene to the short arm of chromosome 19, and identified a two-centimorgan critical region containing the CADASIL gene⁵. In the new paper, they describe how they constructed yeast and bacterial artificial chromosome contigs encompassing the critical region, and isolated transcripts from this region by selection of complementary DNA. One of the isolated clones was homologous to the mouse *Notch3* gene⁶, and they examined this as a candidate for the CADASIL gene. The analysis showed that there were ten different missense mutations in the *Notch3* genes of 14 unrelated CADASIL patients.

Proteins belonging to the Notch family are transmembrane receptors that contain several conserved peptide motifs (see figure). The extracellular domains contain many tandemly repeated copies of an epidermal growth factor (EGF)-like motif. The intracellular domains contain six copies of another conserved motif, termed the Cdc10/ankyrin repeat. Both the EGF and the ankyrin-repeat motifs are found in many different proteins and, in at least some cases, they have been shown to be

involved in protein-protein interactions.

The types and locations of the mutations detected in the *Notch3* gene of CADASIL patients are revealing. All of them were missense mutations, and nine of the ten either added or mutated a cysteine residue in one of the EGF-like repeats. The tenth mutation altered a conserved glycine residue in one of the Cdc10/ankyrin-like repeats. These mutations, particularly those in the EGF-like repeats, would be expected to strongly



Mutations identified by Tournier-Lasserre and co-workers³ in the human *Notch3* gene of CADASIL patients, compared with *Abruptex* mutations in the *Drosophila Notch* gene. Arrowheads represent the site of a missense mutation, most of which are found in the extracellular EGF-repeat domain (purple), although one of the CADASIL mutations is in the intracellular ankyrin-repeat domain (green). The *Abruptex* mutations show a more clustered distribution than do the CADASIL mutations. (Sequence data on the *Abruptex* mutations were taken from ref. 11.)

affect protein conformation. However, an important question is what effect they have on the function of the protein encoded by the *Notch3* mutant allele.

As its name states, CADASIL is inherited in an autosomal dominant pattern. So the alterations in the *Notch3* gene that produce the symptoms of CADASIL must either result from a gain-of-function mutation or they must be due to haploinsufficiency of a *Notch3* null mutation — a haploinsufficiency phenotype results when a single copy of a gene is not sufficient for normal function. At present there are not enough data to decide between these two alternatives. It may be informative, however, to examine mutations in the Notch-family genes of other organisms. For example, in *Drosophila*, the *Abruptex* (*Ax*) mutations are a cat-

egory of dominant mutant alleles of *Notch*. As in the human *Notch3* gene, the *Ax* mutations are missense mutations in the region that encodes the EGF repeats, but these mutations do not create null alleles of *Notch* — instead, they seem to cause ligand-dependent hyperactivation of the Notch protein^{7,8}.

Whether the *Notch3* mutations found by Tournier-Lasserre and co-workers³ in the CADASIL patients cause a loss or gain of *Notch3* function could have important clinical implications. Most of the mutations associated with the CADASIL syndrome cause amino-acid changes in the extracellular domain, so this region of the protein is an obvious target for potential drug development. Studies with transgenic and knockout mice should help to elucidate the effects of these *Notch3* mutations.

The Notch signalling pathway may also be involved in another age-related dementia syndrome — Alzheimer's disease. In a paper published last year⁹, Iva Greenwald's group characterized a suppressor of a gain-of-function mutation in the *lin-12* gene, which encodes a Notch-family receptor in the nematode *Caenorhabditis elegans*. Molecular analysis of several alleles of this suppressor revealed that they were mutations in a hitherto unknown gene, *sel-12*, which encodes a transmembrane protein that is highly homologous to the mammalian presenilin genes: the two presenilin genes, *PS-1* and *PS-2*, are responsible for early-onset familial Alzheimer's disease (reviewed in ref. 10).

Over the past year, lights have been burning late in both academic labs and pharmaceutical companies as researchers try to determine whether alterations in the Notch signalling pathway are involved in the pathogenesis of early-onset familial Alzheimer's disease. Now, more work remains to be done to understand how mutations in the *Notch3* gene lead to CADASIL. The results promise to be both scientifically interesting and, at the same time, important for human health. □

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Leaving no stone unburned

Kevin Zahnle

PERHAPS the earliest widely held theory for the Tunguska explosion was that the world was about to end. As the minutes passed, this theory was dropped in favour of other, less final theories, until today one is hard-pressed to find anyone who truly believes that the world ended on the morning of 30 June 1908. It is now accepted that the explosion marked the end of another world, a small (50–100 m) comet or asteroid destroyed by aerodynamic forces some 10 km above the trees. Yet, because no indisputable remnants of the body have been recovered, the Tunguska event retains an air of mystery. The paper by V. V. Svetsov on page 697 of this issue¹ may explain what happened to the fragments.

The Tunguska impactor exploded above a sparsely inhabited region in central Siberia with the force of a 15 megaton bomb (roughly equivalent to man's biggest). The blast wave flattened trees over 2,000 km² and excited a magnitude-five earthquake. Thermal radiation scorched trees and set fires over much of this range, and even 70 km away an observer removed his shirt for fear it would ignite².

The earliest scientific expeditions to the impact site, launched almost two decades after the event, concentrated on the search for meteorites³. But no meteorite was found. Instead the explorers found a huge region of trees felled in a striking radial pattern, at the centre of which they found standing trees stripped of branches. Apparently the meteor exploded in the air. Although even more interesting theories have been suggested, recent attention has focused on whether the Tunguska body was a comet or an asteroid.

At present, there are two reports of possible debris. One is a modest iridium excess in local peat⁴; the other a relatively high abundance of microscopic dust particles in tree resins exposed between 1902 and 1920 in local conifers that survived the explosion⁵. These may prove to be extraterrestrial, but neither is likely to determine the type of impactor.

Comet partisans argue that an asteroidal Tunguska would inevitably have left large meteorites scattered around the impact site. Comets, it is presumed, would not. Other arguments include a possible genetic link to the Taurid meteors and to comet P/Encke, based on orbital similarities⁶, and the suggestion that the 'white

nights' over Europe that followed the explosion were caused by Earth sweeping up the comet's tail.

Asteroid partisans argue that the Tunguska object penetrated the atmosphere so deeply that, when it finally did explode, it did so while subjected to much higher aerodynamic stresses than those experienced by smaller meteors^{1,6,7}. The higher stresses broke it into smaller pieces. For reasonably strong rock, Svetsov estimates that typical fragments would have been 1–3 cm across. But very few of these 1–3-

IMAGE
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'Ground zero' of the Tunguska explosion, photographed in 1927. Where is the debris?

cm meteorites would have reached the Earth—rather, they were just small enough to have been ablated by thermal radiation in the fireball. An important assumption is that the ablated products are mostly melt droplets, rather than vapour—this makes ablation relatively efficient. All that remains is a spray of melt droplets borne on blast waves.

A difference between this work and earlier work (ref. 7, for example) is its markedly higher luminous efficiency. As Svetsov considers only the opacity of hot air, it is unsurprising that he deduces a luminous efficiency (~30%) like that of a nuclear explosion of comparable yield. This is much higher than for typical meteors, which are optically thin and have luminous efficiencies <1%. Svetsov neglects the opacity of dust and vapours from the impactor in calculating the thermal radiation field. Air must be heated to 6,000 K before it becomes opaque to visible light, but ablated vapours can be opaque at lower temperatures. Thus the effective radiating temperature of an impact fireball is probably less than for a nuclear fireball, and the luminous efficiency (and ablation rates) somewhat less than what Svetsov computes.

It is harder to explain away the iridium.

Extraterrestrial iridium was the tell-tale clue (the 'smoke', as it were) that an impact killed the dinosaurs. The Greenland and Antarctic ice sheets record the background iridium accretion rate, and no additional signal is seen on or around 1908 (ref. 8). If a chondritic or cometary Tunguska had been uniformly spread over the northern hemisphere, one would expect the iridium signal that year to be 10–100 times background⁸.

The surest way to hide iridium is not to supply it, which is possible if the impactor is an achondrite—these are uncommon stones that were once part of a differentiated parent body, in which some regions became depleted in heavy elements. Otherwise, the ejecta must not reach Greenland. They could be launched into space, or broadcast over a limited (~1,000 km) region. A third possibility recalls the white nights over northern Europe. These began on the night of the Tunguska event, and were presumably caused by sunlight illuminating particles of some kind in the upper atmosphere⁹. Unlike volcanic aerosols, the cloud dispersed after two or three days. The particles either evaporated (if ice) or fell (if dust). If the latter, the stratospheric residence time was too brief for the particles to spread out, and so precipitation may have been patchy.

Comet Shoemaker-Levy 9 also exploded in an atmosphere (Jupiter's), so one might hope to draw some analogies to Tunguska. The ejecta blankets extended many thousands of kilometres from the impact site, preferentially along the wake. Ejecta velocities were 10–15 km s⁻¹. Scaling considerations⁹ suggest that Tunguska ballistic ejecta travelled 3–10 times slower, that is 1–5 km s⁻¹, which implies that they would have fallen within 2,500 km of the impact site. At these velocities there is no escape, and so white nights may have depended in part on mesospheric winds. The analogy also suggests that the ejecta were mostly launched towards Manchuria, as the Tunguska impactor came from the southeast. ►

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Small impact craters on Earth are almost always produced by the relatively rare iron meteorites¹⁰. The 1.2-km Meteor Crater in Arizona, for example, was produced by an iron body of essentially the same energy as the Tunguska explosion. The smallest crater known to be made by a chondrite is the 3.4-km New Quebec crater¹⁰. This raises a problem for the comet theory: if comets with energies of 15 MT can reach the troposphere before

exploding, then the much more numerous stony asteroids, which all models agree will penetrate deeper, should be cratering the land every 1,000 years. If Tunguska was a comet, where are all the Meteor Craters made by rocks? □

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RIBOSOMAL RNA

Click here for methylation

Ted Maden

MANY of the nucleotides in eukaryotic ribosomal RNA become modified soon after transcription in the nucleolus (Fig. 1a). A long-standing enigma has been how these particular nucleotides come to be accurately recognized for modification and what the functions of these nucleotide modifications are. Work in Jean-Pierre Bachellerie's laboratory¹⁻³, culminating in a paper on page 732 of this issue⁴, illuminates the process of recognition and opens up a new and precise experimental approach for studying function.

Ribosomes are the universal cellular machines for protein synthesis. Around 50–60 per cent of their mass is rRNA, amounting to some thousands of nucleotides. Certain regions of the rRNA mediate particular functions in protein synthesis, such as the binding of messenger and transfer RNA and (most probably) catalysis of peptidyl transfer⁵. Consistent with these central functions, parts of the rRNA sequences and much of the secondary structure are phylogenetically highly conserved. But there are variations on the conserved theme—for example, the kinds and numbers of modified nucleotides. Eukaryotic rRNA generally contains more modified nucleotides than does prokaryotic rRNA, and nucleotides in which ribose is modified by 2'-O-methylation (Fig. 1b) are particularly abundant. Of the 7,000 nucleotides of rRNA found in human ribosomes, about 105 are 2'-O-methylated. Several other vertebrates contain a similar number of 2'-O-methyls, and the yeast *Saccharomyces cerevisiae* contains about 55.

From work in the 1980s, some overall conclusions emerged concerning rRNA methylation in general and 2'-O-methylation in particular^{6,7}. All of the methylated nucleotides were found within the conserved core of rRNA secondary structure. Within this core distribution, however, there was no consensus sequence or local secondary structure that might serve as an obvious recurring target for methylation. Whereas 2'-O-methylation could occur at a given site in the rRNA of one species, it

was not always observed at the corresponding site in the rRNA from another (usually distantly related) species. This was in spite of the fact that the primary and secondary structures at that site seemed to be highly conserved. For example, several 2'-O-methyls in human rRNA were at sites that were conserved, but unmethylated, in yeast.

So how is 2'-O-methylation directed to specific nucleotides in rRNA? One approach in studying this question is to isolate the nucleolar enzyme(s). Using an unmethylated *in vitro* transcript as the

substrate, Segal and Eichler⁸ partially purified an enzyme which correctly 2'-O-methylated a unique cluster of three consecutive nucleotides in the 28S rRNA of the large ribosomal subunit. However, other sites that are methylated *in vivo* did not become methylated *in vitro* by this approach.

Only recently has there been further progress, starting with the discovery that eukaryotic ribosome biosynthesis involves many small nucleolar RNA (snoRNA) species (see ref. 9 for review). Bachellerie and co-workers¹ drew attention to a class of snoRNAs, isolated from vertebrates or yeast, that have long (10–21 nucleotide) regions of perfect complementarity to highly conserved sequences in 18S or 28S rRNA. These snoRNAs are flanked by consensus sequences called C and D boxes, which allow them to interact directly or indirectly with the nucleolar protein, fibrillarin. Some of the snoRNA species are generated by release from introns during the processing of pre-mRNA for various protein-coding genes. Moreover, in several instances, the complementary rRNA sequence carries a 2'-O-methyl group, characteristically located opposite the fifth nucleotide upstream from a D box in the snoRNA (Fig. 2). This raised a new question: are intron-encoded snoRNA species involved in targeting sites for 2'-O-methylation in rRNA?

To explore this possibility, Kiss-László *et al.*² devised an ingenious method for cloning other—hitherto unknown—species of intron-encoded snoRNAs. They exploited the fact that intron-encoded RNAs have 5' monophosphate and 3' hydroxyl ends, which can be ligated to a ribooligonucleotide, allowing reverse transcription and amplification of the resultant DNA by the polymerase chain reaction. Using this method, 21 new RNA species were identified, each complementary to one or (in two instances) two conserved regions in 18S or 28S rRNA, with each complementary region encompassing a 2'-O-methyl (Fig. 2). Further snoRNAs described in the literature also fitted the pattern, and the combined results² revealed 35 mammalian and eight yeast antisense RNAs with complementarity to 47 2'-O-methylation sites—including nearly 40 per cent of the total mammalian sites. Even more matches were discovered starting

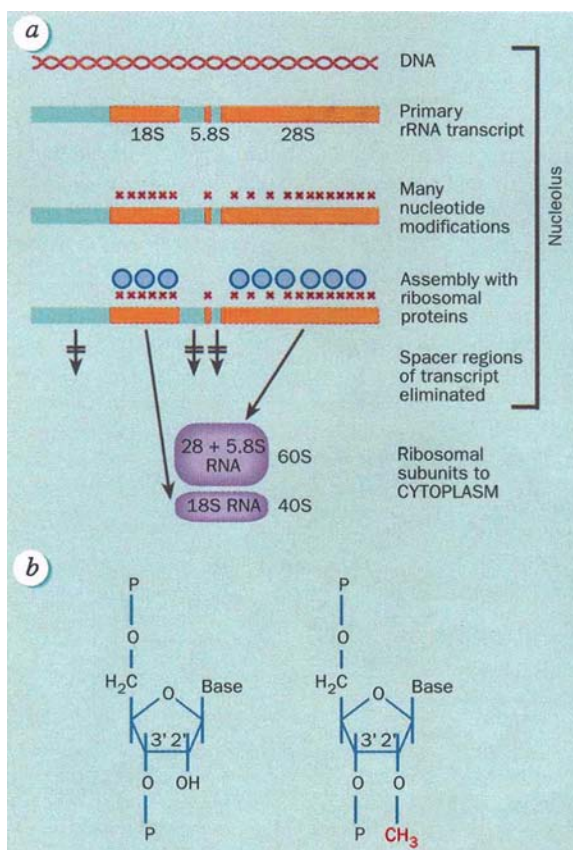


FIG. 1 a, Scheme of ribosome biosynthesis in the nucleolus. There is temporal overlap between some of the events. b, An unmodified nucleotide (left) and a 2'-O-methyl ribose nucleotide (right) in RNA.

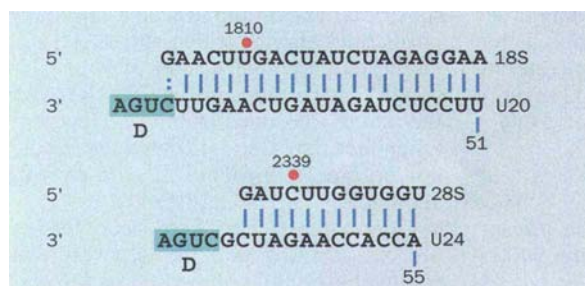


FIG. 2 Two interactions between snoRNAs and complementary sequences in rRNA: U20 pairs with 18S rRNA near its 3' end to specify uridine 2'-O-methylation (red dot); U24 pairs with 28S rRNA near the middle to specify cytidine 2'-O-methylation (and also interacts separately with an adjacent 28S site, not shown). In each case, the methyl group occurs on the rRNA nucleotide opposite the snoRNA nucleotide that is five bases upstream from the indicated D box. (For further interactions see refs 2, 3.)

from a sequence database search³, and each match fitted the 'D box plus five' rule for the snoRNA nucleotide opposite the rRNA 2'-O-methyl. In cases where two 2'-O-methyls were next-but-one neighbours on the rRNA, two separate antisense snoRNAs were found which matched the overlapping tracts encompassing the two rRNA methylation sites.

Stimulated by the impressive correlation between intron-encoded snoRNAs and rRNA 2'-O-methyls, Bachelierie's group went on to obtain experimental support for a causal connection. One snoRNA, called U24, contains two tracts of complementarity to two adjacent stretches in 28S rRNA, each of which is methylated. When U24 is genetically depleted in yeast, methylation does not occur at these sites², but it does occur at other 28S sites. When the U24 gene is reinserted into the intron of a different gene from its original host gene, methylation is restored. When the D box is shifted by deleting one adjacent base, methylation occurs one nucleotide downstream from the normal site. Together these findings² clearly implicate U24 as a precise guide for 2'-O-methylation. One can envisage that snoRNAs pick out target nucleotides for methylation as if 'clicking' letters in a string of text.

Cavaillé *et al.*⁴ have now taken the analysis still further. They have engineered the snoRNA, U20, so that it no longer pairs with its normal target site in 18S rRNA. Instead, one construct now pairs with a new, normally unmethylated site in 18S rRNA. Another construct pairs with a normally unmethylated site in 28S rRNA. The engineered U20s direct site-specific methylation at the new locations. For efficient methylation the D box needs to be adjacent to a region of perfect complementarity of at least 12 base pairs with rRNA. The target nucleotide in rRNA is near to the middle of a complete helical turn, on the same side of the helix as the D box, but on the complementary, rRNA, strand. The arrangement generates a 'docking plus

target' site for the putative methyl transferase. Finally, a U20 construct can be further engineered so that a non-ribosomal 'minisquence' can be methylated after transcription by the normal ribosomal RNA polymerase I, or even after transcription by RNA polymerase II (albeit with a lower efficiency of methylation).

Early studies on mammalian cells indicated that methylation is essential¹⁰, or at least important^{11,12}, for ribosome maturation. However, more recent studies with a yeast temperature-sensitive fibrillar mutant have shown that methyl-deficient ribosomes continue to be formed in this strain, although the growth rate is slowed¹³. Taken together with the new work, these findings indicate that the association of fibrillar with snoRNAs is a prerequisite for the snoRNAs to perform their guide function in methylation. Nevertheless, the precise role of ribose methylation in ribosome maturation remains to be clarified. The new findings by Bachelierie's group will facilitate investigations into the importance of the many methylations on an individual site-by-site basis — for example, when U24 was engineered so as to slightly displace a normal methylation site in 28S rRNA, growth rate was appreciably slowed². Finally, the discovery of a need for guide snoRNAs for 2'-O-methylation should aid in the search for the elusive rRNA 2'-O-methyl transferase enzyme(s), begun promisingly by Segal and Eichler⁸. □

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Gas therapy

THE foot-soldiers of our immune system are the phagocytes, the white blood cells which attack foreign cells or organic matter. Their call to arms is the inflammatory response, and their weapons are oxygen radicals, of which the sharpest and deadliest is the hydroxyl radical. This ferocious molecule oxidizes and wrecks almost any organic molecule it encounters. But like other soldiers, the phagocytes can damage their cause by being too indiscriminately destructive. Inflammation can be so intense as to be a disease in itself. In some cases it is entirely misguided, an assault on the normal tissues of the body. Then it has to be countered with drugs.

Daedalus now proposes a new anti-inflammatory strategy. He wants to counter the oxygen radicals generated by the phagocytes. The obvious counter is a strong reducing agent. The simplest and safest reducing agent, says Daedalus, is hydrogen. It reacts instantly with hydroxyl radicals, and the ultimate product is, of course, simply water. Even better, unlike the standard anti-inflammatory drugs, hydrogen has no mammalian biochemistry and almost no side-effects. Deep-sea divers breathe it safely as a component of their special high-pressure gas mixtures; some report a mild but pleasant narcosis. It could be taken for long periods of time, and when discontinued, would rapidly diffuse out of the body, mainly on the breath.

Not many drugs are gases, so hydrogen therapy poses some novel delivery problems. Simply breathing the stuff would work, but might be hazardous; mixtures of more than 4% of hydrogen in air are violently explosive. It is probably better to sneak it into the body in solution.

Carbon dioxide, of course, is ingested in vast quantities in this way, in fizzy drinks. It can even be occluded in sugar crystals, as in the 'fizzy sweet' Space Dust. DREADCO chemists are working on a soft drink pressurized with hydrogen, and even a hydrogenated fizzy champagne (the gas is more soluble in alcohol; with luck, the hydrogen narcosis will add to that of the alcohol). They are also melting sugar under many atmospheres of hydrogen, hoping to trap a useful amount of it in the solidified sugar. If it works, the product will be formed into pills and tablets. They are even experimenting with a hydrogen poultice to be placed on the site of inflammation. A pilot version contains zinc and hydrochloric acid, but milder chemical sources are being sought. Hydrogen diffuses so rapidly through skin, especially skin swabbed with alcohol, that local inflammation could be swiftly subdued. David Jones

Suntanning in hammerhead sharks

SIR — For many organisms, exposure to high-intensity solar ultraviolet radiation is detrimental^{1,2}. Integumental pigments such as melanin, however, may afford protection from these wavelengths³. We found that the integumental melanin content in juvenile hammerhead sharks increases in direct response to increases in solar radiation. Although humans and some terrestrial mammals 'tan' in response to increases in ultraviolet radiation⁴, this has never been documented for aquatic vertebrates.

Many marine organisms possess integumental melanin, but this pigment is thought to be maintained at constitutive levels and used primarily for purposes other than solar ultraviolet radiation protection. Many marine fishes can rapidly mobilize pigment to facilitate crypsis or to display behavioural cues, but little is known about the photoprotective role of this pigment in aquatic vertebrates^{5,6}. Several studies have shown that some fishes are sensitive to ultraviolet and can experience severe epidermal tissue damage from the sun⁷.

Kaneohe Bay, Hawaii, is a nursery ground for the scalloped hammerhead shark (*Sphyrna lewini*) where pups spend most of their time near the bottom, in the deeper, murky portions of the bay⁸. The pups are light tan in colour but change to dark brown/black over several weeks when held in a shallow (1-m deep) seawater pond with a whitish coral sand/rubble floor. Sharks in the pond experience levels of ultraviolet 600 times greater than at the bay floor, at a depth of 15 m.

To determine if shark pups were 'suntanning' in response to increases in solar

ultraviolet, we exposed sharks in the pond to three spectral irradiance treatments using filters attached to their pectoral fins: (1) ultraviolet-blocking filter, which blocks wavelengths up to 390 nm but allows transmission of visible light; (2) opaque filter, which blocks all wavelengths of light; (3) no filter, which allows full solar exposure (Fig. 1a). We determined changes in melanin densities spectrophotometrically from solutions of skin melanin extract⁹, and found that skin exposed to direct sunlight in the pond significantly increases in melanin concentration by $14 \pm 6\%$ ($n=10$) over 21 days, and by $28 \pm 6\%$ ($n=3$) over 215 days (Fig. 1b). Skin shielded from both visible and ultraviolet light shows no significant change in melanin concentration; distinct pigmentation delineation occurs where the filters cover the skin. Significant melanization occurs only where sunlight makes direct contact with the skin; ventral surfaces of the sharks' skin remain white.

Histological sections of skin from pre-treatment sharks show a low degree of melanization, whereas sharks that were fully exposed to sunlight in the pond showed a marked increase of integumental melanin (Fig. 2). Spectral transmittance through melanin extraction solutions taken from skin of tanned sharks shows the greatest reduction in the ultraviolet-B region (Fig. 1b).

Extensive 'tanning' in response to increased solar radiation may have an adaptive role in protecting against the formation of thymine dimers caused by ultraviolet-B radiation (280–320 nm)¹⁰. It is possible that suntanning is used when

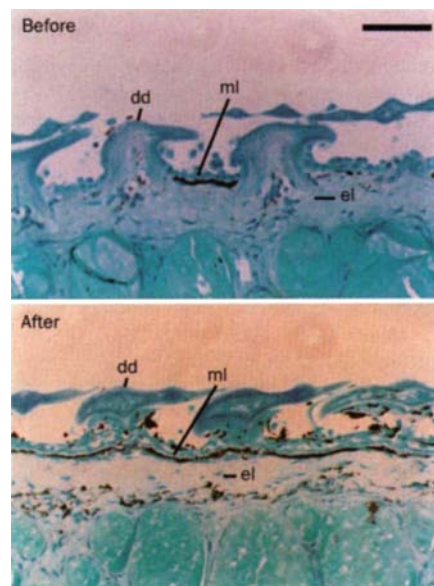


FIG. 2 Cross-sections of hammerhead shark skin from pectoral fin samples stained with methylene blue (sections by J. Wyffels), before (day 0) and after being in the pond for 21 days and exposed to increased solar radiation. dd, Dermal denticle; ml, melanin layer; el, epidermal layer. Scale bar, 50 μ m.

the shark pups leave the bay and enter the clear pelagic waters they occupy as adults.

These findings offer support for the photoprotective role of melanin in aquatic vertebrates. This shark model may have future biomedical applications, because melanomas and dermal carcinomas are unknown in sharks¹¹. Finally, these observations are important for the assessment of biological responses to global increases in ultraviolet.

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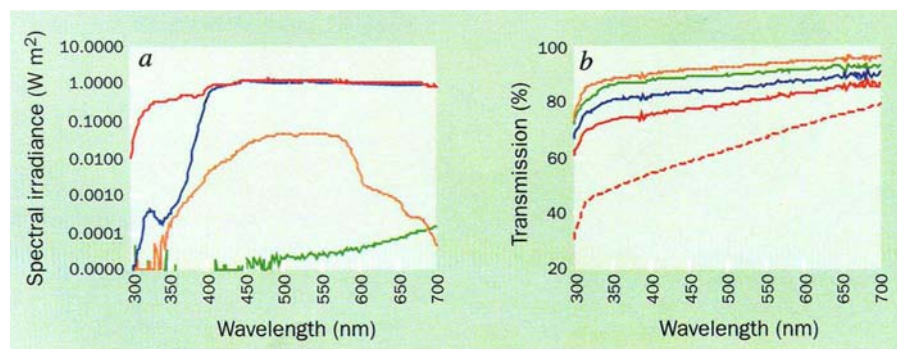


FIG. 1a, We used a LICOR LI-1800 underwater spectroradiometer to measure spectral irradiance (300–700 nm) at the bay floor (15 m depth; orange line); 1 m deep in the shark pond (red line); and through opaque (green line) and ultraviolet-blocking filters (blue line). To determine maximum dosage levels, measurements were made only at midday, on clear days at the beginning of the experiments. b, Mean percentage of transmission of light through melanin extraction solutions from sharks' skin before and after receiving varying spectral irradiance treatments. All solutions were extracted from a 5-mm-diameter plug of skin taken from the dorsal trailing edge of the pectoral fin. Orange line, skin taken from recently captured shark pup from the bay floor; green line, skin shielded by the opaque filter; blue line, skin under the ultraviolet-blocking filter; red line, skin fully exposed to ambient sunlight for 21 days; dashed red line, a shark fully exposed for 215 days. Line colours in b correspond to treatments in a.

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Human homologues of yeast helicase

SIR — Bloom's syndrome and Werner's syndrome are two rare human genetic diseases characterized by genomic instability and a predisposition to cancer¹. The genes responsible for these syndromes have recently been cloned^{2,3}; both encode large proteins similar in size and sequence to each other and to the yeast Sgs1 protein. All three proteins are closely related in a central domain to a known DNA helicase, the *Escherichia coli* RecQ protein. Here we demonstrate that yeast Sgs1 protein has DNA helicase activity. Surprisingly, site-directed mutations that eliminate helicase activity can still complement certain yeast *sgs1* mutants. These results suggest that Sgs1, and by inference, its human homologues, has an important function not related to its helicase activity. In the case of Werner's syndrome, the sites of the sequenced mutations are consistent with this idea.

Two-hybrid experiments indicate that yeast Sgs1 protein interacts with DNA topoisomerases II and III^{4,5}. Yeast *sgs1* mutants were first isolated on the basis of their ability to suppress the slow growth defect of strains lacking DNA topoisomerase III⁴. Yeast *top3* mutants not only grow slowly but also have elevated levels

of mitotic recombination, particularly between repeated sequences⁶. These observations suggest that the Sgs1 family of proteins may be involved in controlling genomic stability and somatic recombination from yeast to humans.

While examining the phenotypes of spontaneous yeast *sgs1* mutants, isolated on the basis of their ability to support the growth defect of a *top3* mutant, we noticed that an *sgs1-top1* mutant grows poorly. Using such a mutant, we cloned the *SGS1* gene by complementation of the poor growth phenotype. As expected, the *SGS1* gene, when introduced into yeast on a plasmid, greatly decreased the growth rate of an *sgs1-top3* mutant (*c* in the figure) and also improved the growth rate of an *sgs1-top1* mutant.

To test whether Sgs1 has DNA helicase activity, we synthesized the protein *in vitro* using a rabbit reticulocyte coupled transcription/translation system programmed with *SGS1* DNA. We used a strand-displacement assay that detects helicase activity and also reveals the polarity of the enzyme. A lysate programmed with DNA encoding a known 5' to 3' helicase, Pif1 (ref. 7), displaced a 40-nucleotide fragment, as predicted (*a* in the figure). In

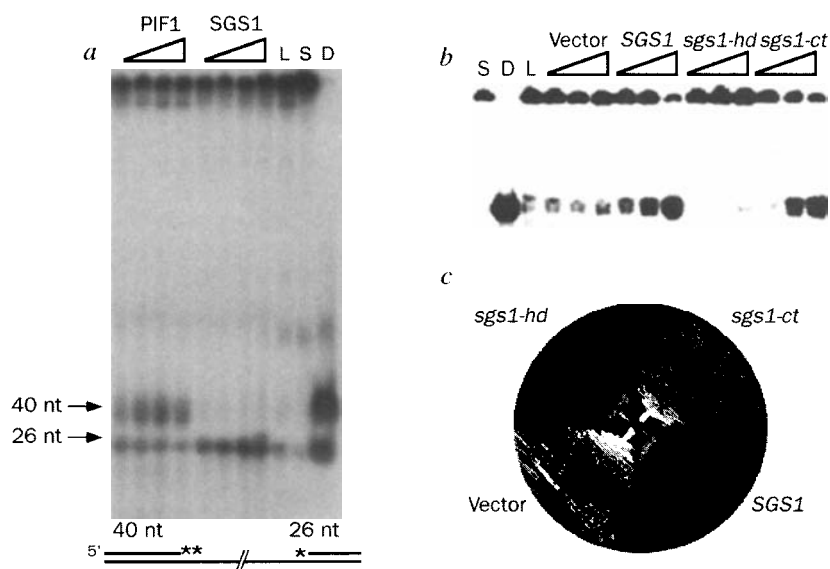
contrast, a lysate programmed with *SGS1* DNA resulted in displacement of a 26-nucleotide fragment. Thus, *SGS1* encodes a 3' to 5' DNA helicase with the same polarity as its bacterial homologue, RecQ⁸.

To inactivate the DNA helicase activity of Sgs1, we mutated the invariant lysine within the ATP binding loop⁹, as this residue is essential for activity in other DNA helicases¹⁰. We used this helicase-defective allele (*sgs1-hd*) to programme the transcription/translation system, and assayed for helicase activity. As expected, we observed no helicase activity (*b* in the figure).

To test the *in vivo* role of the Sgs1 DNA helicase activity, we examined whether a plasmid with the *sgs1-hd* allele could complement an *sgs1* null mutant. Because this mutant grows normally in our strain background, we used *sgs1-top3* and *sgs1-top1* mutants for complementation tests. Surprisingly, the *sgs1-hd* allele behaves just like wild type: it decreases the growth rate of *sgs1-top3* mutants (*c* in the figure) and improves the growth of *sgs1-top1* mutants. We constructed two other mutations in the ATP binding domain of Sgs1. Both behave exactly as seen for the *sgs1-hd* allele. Thus, the DNA helicase activity of Sgs1 is not required for *in vivo* function, at least in these two mutants.

We introduced a linker mutation into *SGS1*, effectively truncating the protein so that it had 1,245 amino acids, compared to the normal 1,447. As expected, this C-terminal truncation (*sgs1-ct*), which is downstream of the helicase domain, exhibits helicase activity (*b* in the figure). When introduced into an *sgs1-top3* mutant, the *sgs1-ct* allele is unable to complement the *sgs1* null mutation (*c* in the figure). The failure to complement is not due to instability of the protein, as the same allele could complement an *sgs1-top1* strain. Thus, the C-terminal domain of Sgs1 protein is essential for function in the *top3* mutant background.

How do these findings relate to Bloom's and Werner's syndromes? Although many Bloom's mutations truncate the protein upstream of the helicase domain, all characterized Werner's mutations truncate the protein beyond the helicase domain. These Werner's mutations probably retain DNA helicase activity just as the *sgs1-ct* allele does. We suggest that



SGS1 encodes a 3'-to-5' DNA helicase. *a*, We programmed reticulocyte lysates with either no DNA (L) or increasing amounts of pT7-PIF1 or pJL34, which can express Pif1 and Sgs1, respectively, from a T7 promoter. We incubated 2 μ l of each reaction with the bidirectional substrate, shown below the gel, and resolved the products by 12% PAGE. Substrate before (S) and after (D) being denatured by boiling; nt, nucleotide. *b*, Helicase assays of *sgs1-hd* and *sgs1-ct* alleles. We programmed lysates with either no DNA (L) or increasing amounts of either pET11a (Vector), pJL34 (*SGS1*), pJL40 (*sgs1-hd*) or pJL38 (*sgs1-ct*) and assayed them as in *a* except we used a [³²P]5' end-labelled 64-mer oligonucleotide annealed to M13mp19 single-stranded DNA as substrate. *c*, Complementation tests of *sgs1-hd* and *sgs1-ct*. We transformed strain AMR60 (*sgs1-3::TRP1*, *top3-2::HIS3*) to leucine prototrophy with pRS415 (Vector), pJL31 (*SGS1*), pJL37 (*sgs1-hd*) and pJM502 (*sgs1-ct*). We streaked transformants onto a plate lacking leucine and photographed them after growth for 2 days at 30 °C. We constructed the *sgs1-3::TRP1* allele by replacing the DNA between the *StuI* site at amino-acid 42 and the *PstI* site at amino-acid 1,046 with a fragment containing the *TRP1* gene. We constructed the *sgs1-hd* allele, K706A, by oligonucleotide-directed PCR mutagenesis and *sgs1-ct* by insertion of the nonsense-containing oligonucleotide CTAGCTAGCTAG at the unique *XbaI* site (amino-acid 1,245) in *SGS1*.

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the genomic instability and subsequent disease symptoms caused by both Bloom's and Werner's mutations may be due to a loss of a function other than the helicase activity, just as is the case for *SGS1*.

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Purifying single-walled nanotubes

SIR — Single-walled carbon nanotubes^{1–3} hold great promise for both fundamental understanding and potential applications of carbon materials^{4,5}. Recently, they have been successfully synthesized in large quantities⁶ using composite consumable carbon electrodes mixed with metallic species, such as Fe/Ni and Co/Ni. The single-walled nanotubes synthesized by arc discharge^{1–3} are found to coexist with metals and with various other forms of carbon, and such impurities are a serious impediment to detailed characterization of the properties of the nanotubes. Various purification methods proposed for multi-walled nanotubes have been applied to the single-walled versions⁷, but so far without clear success. We present here a new purification method for single-walled nanotubes that incorporates a hydrothermal treatment⁸ for easy removal of metal particles and carbonaceous materials.

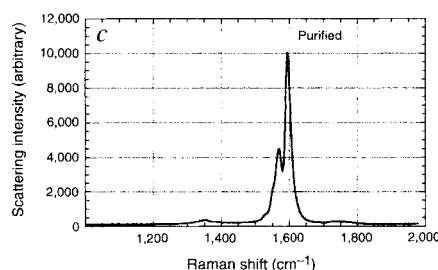
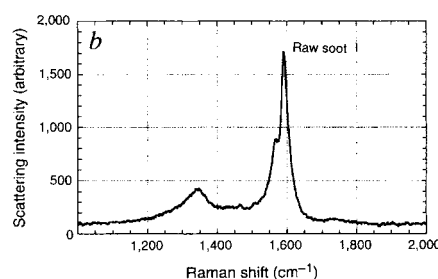
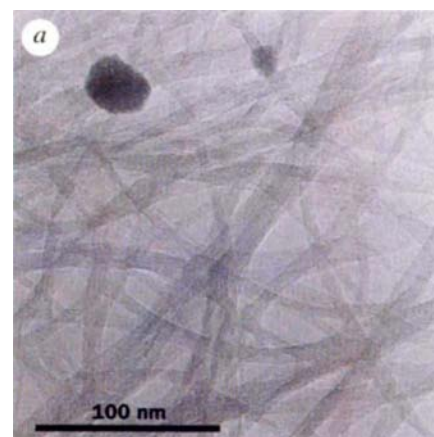
We used the method proposed by Seraphin and Zhou⁶ to produce single-walled nanotubes. The mixing ratio of Ni, Fe and graphite powder was 1:1:3 by weight. The total metal content in the anode is approximately 6 at.%. The arc discharge was carried out under a He pressure of 100 torr. The discharge current was 70 A, and the gap between the

electrodes was maintained at 1 mm by manually advancing the consumed anode.

We retrieved soot produced by d.c. arc discharge from the upper wall and roof of an arc-discharge chamber and homogenized it. Single-walled nanotubes coexist with many by-products in the raw soot, such as metal particles, fullerenes and amorphous carbon. The HIDE treatment⁸ is the initial step of the purification process. We introduced 100 mg of the soot containing the nanotubes into a flask with reflux attachment, sonicated it in 50 ml of distilled water and heated it at 373 K for 12 h. The soot disintegrated to sub-micrometre-sized particles. We filtered out the processed soot and dried it at 333 K for 12 h. Then we washed out fullerenes using toluene Soxhlet extraction. We heated the residual soot at 743 K for 20 min in air to burn out the amorphous carbon. In the final step of the purification process, we washed out almost all metal complexes from the soot using 6 M hydrochloric acid. We obtained at least 20 mg of single-walled nanotubes (with a purity of 95 wt%) per g of raw soot. One gram of raw soot contains roughly 50 mg of unpurified single-walled nanotubes. The purified nanotubes (*a* in the figure) are almost totally free of impurities and are separated in large quantities.

We then qualitatively analysed bulk samples of purified single-walled nanotubes for residual amorphous carbon using Raman scattering. The nanotubes show multiple-split Raman peaks of crystalline graphite centred at 1,580 cm⁻¹ because of their cylindrical symmetry, whereas amorphous or fine graphitic particles exhibit broad peaks around 1,350 and 1,580 cm⁻¹. Our Raman spectrum of raw soot, showing a very broad peak near 1,350 cm⁻¹ together with weak structures between 1,400 and 1,500 cm⁻¹ of fullerene, is shown in *b* in the figure. The Raman profile matches the spectrum reported by Holden *et al.*⁹ very well. Purified single-walled nanotubes are shown in *c*, with a spectrum around 1,580 cm⁻¹ showing multiple-split peaks (1,570 and 1,590 cm⁻¹) that are five times more intense than in soot, without any change in their positions and intensity ratios, and clear shoulders (1,530 and 1,550 cm⁻¹). These results suggest that we have successfully purified undamaged, single-walled nanotubes, and that only trace amounts of other carbonaceous materials remain in our purified sample.

To confirm the role of the HIDE treatment in the purification process, we processed the nanotubes without hydrothermal treatment. In this case, large amounts of graphite and metal particles are associated with them. Further, the surfaces of the bundles are covered with amorphous carbon. This confirms that without hydrothermal treatment ultrafine graphite particles, nanocapsules and amorphous carbon adsorbed on the single-



a, Transmission electron micrograph of purified single-walled nanotubes. *b*, *c*, Raman spectra of raw soot and purified single-walled nanotubes, respectively.

walled nanotubes are not removed from the soot. This may be the reason why the baking technique is not successful. We believe that, during the HIDE treatment, water molecules break the network between single-walled nanotubes, amorphous carbon and metal particles, and also attack the graphite layer encapsulating the metal particles. Consequently, almost all graphite nanoparticles and nanocapsules are washed out from the soot.

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Biological particles over Antarctica

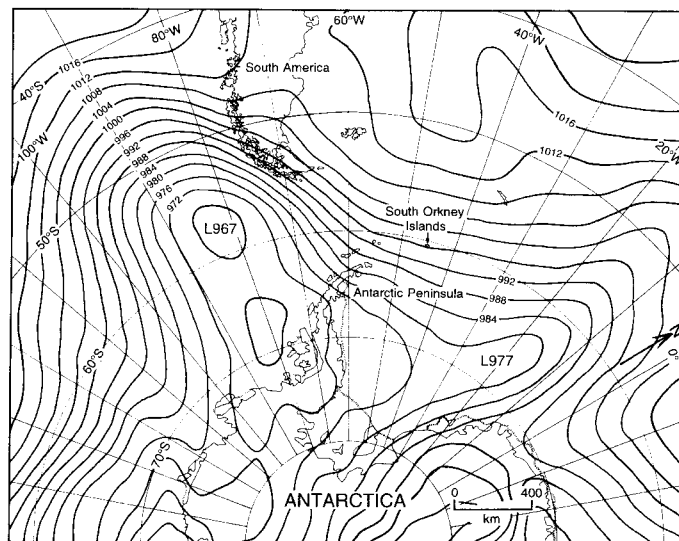
SIR — Antarctica is viewed by almost all as a continent in biological isolation. But recent aerobiological sampling in the maritime Antarctic has revealed a dramatic influx of material from South America when, within a single 24-hour period, the density of the air-spores increased 20-fold over normal levels. This was associated with a specific weather pattern which occurs with an estimated mean annual frequency of 1.5. This is the first time such an impressive transfer of biological material from another continent has been observed and it provides direct evidence of one way in which the Antarctic may have been recolonized since the last glacial maximum, about 18,000 years ago. Such events also provide a mechanism for organisms to extend their range into Antarctica as mean annual isotherms move south with regional warming, as in recent years¹⁻³.

I conducted an aerobiological sampling programme at three sites (111, 150 and 279 m above sea level) on Signy Island, South Orkney Islands in the maritime Antarctic (60° 43' S, 45° 36' W) between 14 December 1992 and 28 January 1994. Samples collected between 9:20 on 11 November 1993 and 11:00 on 12 November 1993 showed a massive increase in concentration of airborne biological particles, 24, 17 and 13 times above the mean for all samples collected at each of the three sites. This was more than three times that of the next largest concentration sampled at two of the sites and 1.6 times that at the third.

However, the concentration of airborne pollen and spores at Signy Island during this period was still much smaller than that at temperate sites (3.34 m⁻³ compared to 12,500 m⁻³ during the summer in southern England⁴). The massive increase in the air-spores at Signy Island represents a small proportion of spores that diffuse upwards beyond a point where they may be at risk of dry deposition in South America and that completed the 1,500-km intercontinental journey between Cape Horn and the South Orkney Islands (see figure).

Exotic pollen records collected over the past 100 years in snow, ice, moss and peat have provided much circumstantial evidence for long-distance transport of propagules into Antarctica. In the present study, I found large concentrations of

southern beech (*Nothofagus* spp.) pollen in the air (mean from three sites, 0.47 grains m⁻³), indicating that the material trapped on Signy Island on 11–12 November 1993 was from South America. Other material, not normally found in Antarctic air, included spores of the widely distributed fungi, *Tetraploa* spp. and *Sporormiella* spp., and pollen of South American *Podocarpus* spp. Examination of daily synoptic weather charts, of the South



Daily synoptic chart for 11 November 1993 showing the weather pattern resulting in the transport of biological material from southern South America to the South Orkney Islands in the maritime Antarctic. Tightly packed isobars indicate a corridor of strong winds between Tierra del Fuego and Antarctica as air flows clockwise around the area of low pressure (L967). Collections were made over 24-hour periods using four rotorod samplers on each of three sites¹⁴.

Atlantic and South Pacific sectors, between 40 and 90° S, for the period November 1983 to October 1995 revealed weather patterns very similar to the event of 11 November 1993 on 18 occasions (mean 1.5 per year).

During the last glacial maximum, ice covered all but the tallest mountain peaks and scoured marine habitats (up to 200 m below the present sea level) as far north as the sub-Antarctic islands^{5,6}. Following this destruction of coastal terrestrial and benthic habitats⁷ two mechanisms of recolonization are possible, the radiation of organisms from ice-free refuges and the immigration of organisms from more

northerly land masses by long-distance dispersal. Ice-free land would be limited to isolated nunataks; pre-glacial relict species in the terrestrial environment are therefore nunatak specialists. In contrast, most organisms inhabiting the maritime and sub-Antarctic are less specialized and possibly arrived by long-distance airborne transport.

Chilean *Podocarpus* spp. pollen found in samples of red snow collected by the Scottish National Antarctic Expedition of 1902–04 (ref. 8) was the first reported evidence of long-distance airborne transport into the Antarctic. Recent examination of moss cushions for exotic pollens has indicated that immigration occurs in the maritime Antarctic but not continental Antarctica⁹. However, this is contradicted by the growth of exotic plants on volcanically warmed ground around fumaroles in continental Antarctica¹⁰. A coordinated aerobiological sampling programme in the Antarctic has been suggested several times since the 1960s (refs 11, 12). Previous studies have been summarized by Smith¹² and Wynn-Williams¹³. In this study I have found conclusive evidence of immigration into the maritime Antarctic, but only further sampling programmes in other regions of Antarctica will determine the full extent of this phenomenon.

The possible frequency of these events represents considerable potential for the input of biological material into the Antarctic biome since the last glacial maximum and, therefore, opportunities for colonization of the increasing snow-free area. If future climates continue to make pristine habitats available for colonization, phenomena like the one described here will be of major importance in defining which taxa are successful.

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Peddling prosperity

Philip Gummert

Science, Technology and the British Industrial 'Decline', 1870–1970. By David Edgerton. Cambridge University Press: 1996. Pp. 88. £17.95, \$29.95 (hbk); £6.25, \$9.95 (pbk).

At the end of a lecture on the decline of Britain as a world power, a colleague was recently accused by a student of being unpatriotic. The lecturer reminded the student that his starting point had been the reign of King Henry VIII. Decline is a relative matter, as David Edgerton, working over a shorter period, labours to establish. That those who were initially strongest find their dominance increasingly challenged by newcomers does not necessarily mean that they are doing all that badly.

In 69 pages of text, Edgerton attempts a lively demolition of what he takes to be a range of orthodox positions on the "problem" of British industrial "decline", and the extent to which that decline is due to failures to invest in, or properly apply, science and technology. His targets include, among many others, C. P. Snow, Corelli Barnett, Martin Wiener and assorted "technocratic" (in Edgerton's usage of the term) writers on science policy, including Christopher Freeman.

Edgerton argues that many of the participants in this debate have confused relative decline with "failure". This, he suggests, has led them to overlook the fact that, until the 1960s, Britain was the second-largest industrial economy in the world, with investment in science and technology to match, and a record in technological innovation at least as good as that of Germany. Suggestions of an anti-science culture in British boardrooms and government are met with statistics about the percentage of company directors with technical backgrounds, or the degree of both industrial and state funding of research and development (R&D).

Useful original data are presented to show that British industrial spending on R&D from the 1930s to the 1970s was much higher than is often assumed, and that most large manufacturing companies were doing R&D by the 1930s, even if the same was not true of smaller companies. He also emphasizes the volume of government spending on R&D, particularly on military R&D, between the world wars. This was dramatically so in the case of aviation: the Royal Air Force in the 1930s had hundreds of aircraft, compared with Imperial Airways having mere tens; the Air Ministry was easily the largest government spender on R&D. Similarly, in the 1940s and 1950s, its successor, the Min-

istry of Supply, spent more than private industry on R&D.

This is a valuable corrective to some of the more simplistic claims that have been made about the centrality of science to wealth creation. But in addressing so large a subject in so short a book, Edgerton is forced to compress brutally his own arguments (even the bibliography is shorn of publishers' names). As a result, they often come across with less subtlety or precision than might be thought desirable. For example, he makes the apparently revisionist claim that "[d]espite the protests of scientists, and the laments of historians of 'science policy', the British state usually spent more on warlike R&D than on civil R&D, and never concentrated its funding of R&D in one department of state [he is referring to the Department of Scientific and Industrial Research]". This not only contains an unfortunate ambiguity about the subject of the protests and laments

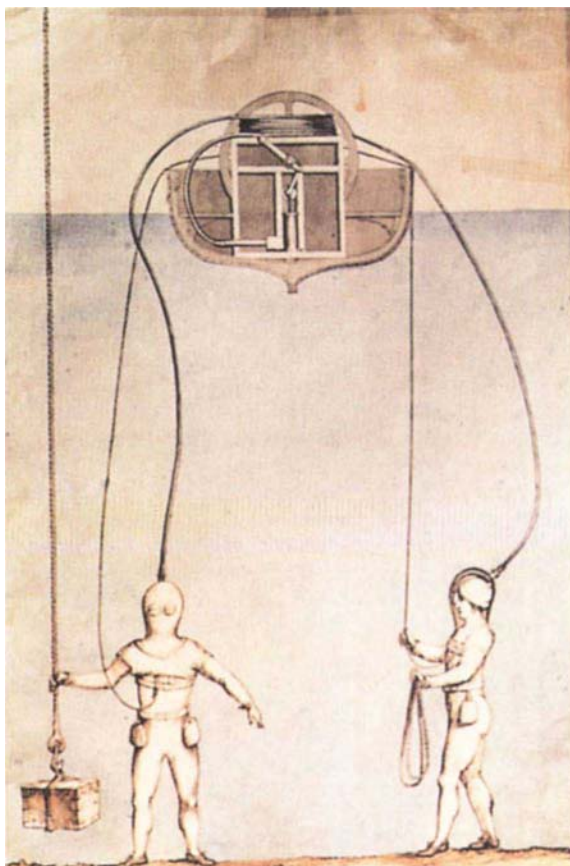
but, more importantly, also seems to overlook material in at least one of the two sources he cites that shows that these historical mistakes were not in fact universally made.

He later challenges the claim by some analysts that the mistakes made over large-scale investments in the 1960s and 1970s in civil high-technology projects lay in their choice of sector (principally aerospace and nuclear power) rather than in the volume of investment itself. He does so by asserting that the trouble was that "government, scientists and engineers all genuinely believed that civil aerospace and nuclear power were the technologies of the future". Really? Not a single doubter among all those people?

This is a pity, because the target audience, "students approaching a subject for the first time, and... their teachers", deserve a more nuanced analysis than the book often manages and which, had more space been available, the author could have provided. So although the book will undoubtedly be of value to them, it needs to be treated with as much care as its author would attach to those he criticizes. □

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"THEIR apparatus was of a very homely character, and appears to have been devised under the necessity which ingenious men in want of capital are apt to experience." In *The Infernal Diver*, John Bevan reinstates Charles and John Deane as the inventors of the diving helmet, seen here in a sketch made around 1830 by Simon Goodrich, chief mechanist of the Royal Navy. In detailing the way in which the helmet was first applied to salvage, treasure hunting, civil engineering and military uses, the author provides insight into the process of technological development in the early nineteenth century — an age when the amateur inventor, working with limited resources, was an important figure in industry. Submex, 21 Roland Way, London SW7 3RF, UK. £60.



Rights from wrongs

Stathis Psillos

Error and the Growth of Experimental Knowledge. By Deborah G. Mayo. University of Chicago Press: 1996. Pp. 493. \$74, £59.25 (hbk); \$29.95, £23.95 (pbk).

How does evidence relate to theory? Karl Popper thought that evidence can exercise only negative control: it can only falsify a hypothesis. Inductivists protested that this distorts the way in which theories are and should be evaluated, claiming that evidence can also exercise positive control: it can confirm a hypothesis in the sense that it can make the hypothesis more probable than it was taken to be before the evidence came in.

There was hope that a solution might be found when Rudolf Carnap attempted to show, based on the probability calculus, that this intuitive idea can be developed into a precise logic comparable to deductive logic. Carnap thought that, in a manner analogous to full entailment, in which whenever the premises are true, the conclusion has to be true, we might have objective degrees of entailment: a piece of evidence confirms a hypothesis by partially entailing it. But 'inductive logic', as it came to be known, was not to last. Applying the probability calculus required that some initial weight should be given to the hypotheses. Any attempt to equi-partition the weight across these hypotheses, based on an intuitively clear principle of indifference, led to paradoxes and incompatible confirmation measures.

The current orthodoxy in confirmation theory takes its cue from Carnap's failures. 'Bayesians', so called because of the central role they attribute to Bayes' theorem of probability calculus, suggest that the degree of confirmation of a hypothesis by some piece of evidence is captured by the difference between two probabilities: the posterior probability of the hypothesis given the evidence, minus its prior probability, its initial weight before the evidence came in. This neat idea offers a general model of scientific inference: on learning the evidence, use Bayes' theorem to update the initial degree of belief in the hypothesis. The posterior probability of the hypothesis thus calculated is the agent's new degree of belief in the hypothesis.

If Carnap's principle of indifference fails, where do the Bayesians' initial weights come from? The standard 'subjectivist' answer is that they express the agent's subjective degree of belief in the hypothesis. Bayesians are not concerned with how the initial probability distribution is determined, but rather with how one's degrees of belief should be redis-

tributed once the evidence is accepted.

Bayesian confirmation theory successfully tackled several problems and paradoxes that plagued its rivals, and so enjoys great respect. But many philosophers of science are naturally unhappy with its reliance on subjective degrees of belief as a means of specifying prior probabilities. Deborah Mayo offers a sustained and systematic critique of this subjectivism. But she also advances an alternative to Bayesianism that rests on the standard Neyman-Pearson statistics and uses error probabilities understood as objective frequencies. Mayo's error probabilities do not characterize the degree of confirmation of a hypothesis; they refer to the experimental process itself, and specify how reliably it can discriminate between alternative hypotheses.

Mayo is very sensitive to the severe criticism that has been directed at the underlying Neyman-Pearson statistics. To get round this, she tries to show either that the criticism has been based on a misinterpretation of the Neyman-Pearson statistics or, where the criticism finds its target (for example, the critics' point that the Neyman-Pearson statistical methods cannot show how a test can provide strong or weak evidential support to a hypothesis), that there are ways to develop the theory so that the objections are met.

Mayo's multi-targeted book weds her error-probabilities approach with the recent work in experiments and scientific model building to provide a general framework of experimental learning. Ian Hacking has shown that experiments have a life of their own, independent of high-level theorizing. What Mayo adds, in an engaging and well-argued way, is that experimental learning also has a life and a model of its own. Experimental learning is intimately connected with severely testing experimental hypotheses using reliable test procedures (those that have a high probability of detecting an error if there is one, and not registering an error if there is not). Experimental learning may be messy and piecemeal, and may require a tool-kit of techniques that ensure low error probabilities, but, as Mayo shows, it can be embedded in a rather neat statistical framework that makes a virtue out of our errors.

There might have been more about how and whether these insights apply to full-blown scientific theories. Mayo is aware of the significance of the problem, but does not discuss it in any detail. Despite this, however, there is a high probability that this important book will make an impact on the scientific community. □

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New in paperback

Engines of the Mind: The Evolution of the Computer from Mainframes to Microprocessors by Joel Shurkin.

Norton, \$13, £9.95. A lively account by a journalist, with a new chapter and epilogue on the latest developments in computing.

Physical Chemistry from Ostwald to Pauling: The Making of a Science in America by John W. Servos.

Princeton University Press, \$19.95, £16.50. A highly praised portrait that shows how science is shaped by personalities, communities and institutions, as well as ideas.

Benjamin Franklin's Science by I.

Bernard Cohen. Harvard University Press, \$17.95, £11.95. An eminent historian of science considers Franklin's ideas and experiments and their influence throughout Europe and the United States.

Scientific Elite: Nobel Laureates in the United States by Harriet Zuckerman.

Transaction, \$24.95. An absorbing study that looks at the interplay between merit and privilege by tracing the careers of all American laureates who won prizes between 1907 and 1972. Contains a new introduction by the author.

The Rhetoric of Science by Alan G.

Gross. Harvard University Press, £11.95. With examples ranging from Copernican astronomy to today's peer review system, the author shows the ways in which scientists try to establish authority and convince others of the importance of the truth they describe.

Hostages of Each Other: The Transformation of Nuclear Safety

Since Three Mile Island by Joseph V.

Rees. University of Chicago Press, \$15.95, £12.75. Takes the reader behind the scenes of nuclear power in an in-depth examination of the dramatic change in the industry's safety standards, operation and management in the wake of the Three Mile Island disaster in 1979.

Theories of Vision from Al-Kindi to Kepler by David C. Lindberg.

University of Chicago Press, \$16.95, £13.50. A close-up look at the development of physical optics as well as our psychological understanding of visual experience.

The Origin of Language: Tracing the Evolution of the Mother Tongue by Merritt Ruhlen.

Wiley, \$16.95. A popular account of historical linguistics.

The Particle Garden: Our Universe as Understood by Particle Physicists by Gordon Kane.

Addison-Wesley, \$12. A short readable introduction for scientists and nonscientists alike.

Flawed conceptions

Yvonne Marshall

The Prehistory of Sex: Four Million Years of Human Sexual Culture. By Timothy Taylor. *Bantam/Fourth Estate*: 1996. Pp. 353. \$23.95, £18.99.

IN this wide-ranging survey of sexual behaviour in human history, Timothy Taylor argues, in opposition to sociobiologists, that human sexuality must be understood as primarily cultural rather than biological. To him, the diversity of sexual practices in prehistory indicates that human sexuality is as much a social activity as a reproductive necessity. Combine this with an attempt to account for the origins of patriarchy, and one might reasonably expect a good read. But all is disappointment.

Just because a book is written for a general audience does not mean it is excused from meeting the requirements of good scholarship. As the successes of Stephen Jay Gould's many books demonstrate, good scholarship sells widely. Although we cannot all aspire to such pinnacles of achievement, adequate referencing of source material, especially when it is contentious or poorly known, and the development of a coherent, theoretically informed argument, constitute a good start. But Taylor neglects these basic requirements, offering instead a series of anecdotal comments that lack authority and fail to develop ideas in any meaningful way.

This is evident from page four where, sandwiched between titillating speculations about whether Ötzi, the Iceman, still has semen preserved in his body and, if so, whether it resides in his scrotum or rectum, we are informed that early hominid females had large clitorises while males "had vanishingly small penises". Thankfully this alarming situation was sorted out in the course of human evolution and the "clitoris reduced in size, while the penis grew dramatically larger". Such a riveting, if dubious, assertion promises a rollicking good story. But, typically, Taylor has little more to tell us and his tossed salad of anecdotes eventually becomes boring.

In pursuit of his objective "to challenge the sociobiologists", Taylor provides summaries of theories he intends to take issue with and quotes extensively from authors he wishes to criticize. But, ironically, without a well developed argument of his own, this has the effect of promoting sociobiological ideas to the reader: his thumbnail sketches of other people's theories seem comparatively convincing placed alongside his own.

Similarly, his attempts to promote feminist perspectives have the opposite effect

because he clearly does not understand the literature on sex, gender and sexuality and cannot even use these concepts consistently, never mind make an original contribution. What's more, relevant work by feminists is ignored or commented on only briefly. One example is the work of Adrienne Zihlman and Nancy Tanner, who suggested in 1976 that the first artefact was probably a bag invented by women for carrying plants and babies. Yet Taylor seems to claim this idea for himself: "I believe that the invention of the baby-sling was the single most crucial step in the evolutionary development toward larger brains." Just one more immaculate conception. In the same way, Marie Louise Sörenson, an archaeologist well known for her work on prehistoric clothing, is never mentioned despite Taylor's frequent use of the idea that clothing transformed sexual relations. Such omissions are numerous.

Epitomizing Taylor's failings is the bizarre interpretation he offers for the image portrayed on a silver-gilt horse harness and reproduced as the cover illustration. In this image, a seated man is shown copulating with a woman astride his lap. Another woman holds in one hand a vessel and in the other a plant that hovers over or between the couple's heads. This scene is usually described as a sacred marriage, and I agree with Taylor that this interpretation takes a fair degree of licence. But his own assertion that "it seems clear that the man is being seduced, even raped", made on the basis that the man must be drugged and



Sacred marriage or strange seduction?

the plant obscures his view of the woman, is simply ridiculous.

The challenge to sociobiology presented by the archaeological evidence of past human sexuality informed by feminist theory is not only real, but also has the potential to transform our understanding of what sex, gender and sexuality mean in human terms. To find it reduced to a series of facile, unfounded assertions is enraging. One can only hope that a more intelligent treatment will appear quickly. □

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Unravelling biological complexity

Nathan Ellis

Human Molecular Genetics. By Tom Strachan and Andrew P. Read. *BIOS/Wiley*: 1996. Pp. 597. £29.95, \$44.95 (pbk).

SPRINGING from classical genetics, human genetics and molecular biology, the nascent field of human molecular genetics was born out of a series of powerful innovations referred to as the recombinant DNA technology. Ever since, the field has been to a large extent driven by new and ever more powerful technological innovations, such as the polymerase chain reaction and the newly developing areas of whole-genome cloning and sequencing, with their dependence on robotics and informatics.

As a field that is both advancing and evolving rapidly, human molecular genetics does not have many, if indeed any, definitive textbooks. This deficiency arises not only from the rate of technological change but also from the sheer volume of new information in this field, which underlines the biological complexity comprehended with the new tools. Textbook authors have

chosen their audiences carefully, and taken their chances by writing about particular aspects.

The theme of this textbook is the structure and function of the human genome, and the authors use the technological developments of the past 15 years as the basis for discussion of this theme. Particularly well done is the interplay between the formal genetic analysis of human disease and the use of modern mapping methodologies, both genetic and physical, to attack what were once considered unsuperable obstacles. The handling of mechanisms and consequences of mutations, of the organization of sequences of the human genome, and of the evolutionary processes that have shaped it, are unique aspects of the authors' approach, setting the textbook apart from others in the area. The emphasis is more on the genome itself than on medical conditions arising from its disturbance.

A technological approach such as this has its dangers. When the complete sequence of the human genome has been

determined, the interest in intermediate technologies, such as chromosome jumping libraries or chromosome walking, becomes more historical. The advantage of such an approach is that it provides the uninitiated with the tools to approach the literature. The book provides an up-to-date and encyclopaedic treatment of these modern technologies, most of this information being very robust. Besides, the day when the sequence of the human genome will be complete is a long way off.

A bias of the technological approach comes from not putting subjects in an adequate biological context. For example, the chapter on recombinant DNA technology would have been clearer if it had been placed in the context of bacterial genetics. From the heuristic viewpoint, such contextual information is valuable because it provides a framework for understanding the capacities and limitations of the technology. From a practical viewpoint, there is only so much the authors can discuss in depth given the limited space. In a sense, students today need to know how to use and interpret these technologies, rather than to know the intricate biology that conceived them. However, the authors might have remedied this bias by taking more advantage of the sections of further reading at the end of each chapter. A criticism along the same lines is that the references in the text often seem rather arbitrary.

The figures, tables and boxes present a large part of the information, and in general they are well designed and extremely useful. The first few chapters would have been improved by more examples of real experimental output, for example showing a human karyotype when treating cytogenetics, and showing an autoradiograph of a Southern blot when treating this hybridization method. Occasionally there is a lack of attention to detail in the legends. These weaknesses notwithstanding, the textbook can be recommended on the value of these presentation items alone.

A book such as this, which encompasses all the major modern technologies in human molecular genetics and their applications, can be used at an advanced level as a course textbook for students, or by mature investigators outside the field who are seeking an epitome of these recent advances. In many ways, it addresses the gap between introductory textbooks and the primary literature. It will no doubt contribute to the education of the young and uninitiated, as a valuable tool in formal training in human molecular genetics. There's no other textbook quite like it. □

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NAVAJO sandstone, Colorado Plateau, Utah, United States. The picture is taken from *Rocks and Fossils* by Arthur B. Busby III, Robert R. Coenaads, Paul Willis and David Roots, a well illustrated guidebook describing a wide range of rocks and fossils, and the sites where they can be found, as well as the history of the Earth and the forces that have shaped it. HarperCollins/Nature Company, £14.99.

Global outlooks

Hazel Rymer

Shocks and Rocks: Seismology in the Plate Tectonics Revolution. By Jack Oliver. *American Geophysical Union*: 1996. Pp. 139. \$28 (pbk).

FOR THOSE of us who came to study Earth sciences during the 1980s, it is hard to imagine a time when the theory of 'plate tectonics' did not exist. It is perfectly appropriate to describe the development of the theory during the 1960s as the great Earth-science revolution.

Before then, the Earth was seen as a sphere with a thick, inert, rocky mantle encasing a central molten, metallic core. The complex pattern of land and sea masses was believed to be essentially static. By the end of the revolution, only the core remained. The mantle had become a solid yet flowing region convecting heat from within the Earth through a thin, strong and brittle shell that was broken into a few large plates moving laterally on and with the mantle. Jack Oliver's description of the role of seismology in this revolution is a highly personal account from the perspective of the Lamont Observatory.

It was Abraham Ortelius, in 1596, who first came up with the idea that North America, South America, Eurasia and Africa were once joined together and have since drifted apart creating the modern

Atlantic in the process. This insight was largely ignored for almost 400 years. What Ortelius lacked was a large body of supporting geophysical evidence and the means to communicate his ideas to a wide audience. These are themes that Oliver continually returns to.

Although earthquakes were well documented by the ancient Greeks and Chinese, it was not until the Cold War, and the need to verify adherence to the Nuclear Test Ban Treaty, that substantial funding — and data — became available for seismologists to study the deep structure of the Earth. It takes more than some new data to make a revolution though, and Oliver illustrates that good communication of results, not just the routine publication of them, is the key to making a piece of scientific work influential. Communication across the various disciplines is the key to this and future revolutions. Oliver demonstrates, with amusing anecdotes that bring the story alive, that good science requires a combination of hypothesis testing, serendipity and sound strategic and tactical thinking — as well as good fortune.

Shocks and Rocks is a thoroughly good read for scientists and interested nonscientists alike. The author uses a real case history to show the way in which science is done, and conveys the excitement of scientific discovery and insight with energy and humour. □

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Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD

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Strains of transmissible spongiform encephalopathies are distinguished by differing physicochemical properties of PrP^{Sc}, the disease-related isoform of prion protein, which can be maintained on transmission to transgenic mice. 'New variant' Creutzfeldt–Jakob disease (CJD) has strain characteristics distinct from other types of CJD and which resemble those of BSE transmitted to mice, domestic cat and macaque, consistent with BSE being the source of this new disease. Strain characteristics revealed here suggest that the prion protein may itself encode disease phenotype.

THE prion diseases or transmissible spongiform encephalopathies are a group of neurodegenerative diseases that affect both humans and animals. They can be transmitted between mammals by inoculation with, or in some cases dietary exposure to, infected tissues. They are all associated with the accumulation in affected brains of an abnormal isoform of a host-encoded glycoprotein, prion protein (PrP), which seems to be the central (and possibly the sole) component of the transmissible agent or prion¹. This disease-related isoform, PrP^{Sc}, can be distinguished from the normal cellular isoform, PrP^C, by its insolubility and partial resistance to proteases. PrP^{Sc} is derived from PrP^C by a post-translational mechanism² which seems to involve a conformational, rather than covalent, modification³. Transgenic, human molecular genetic and *in vitro* conversion studies support a model for prion propagation which involves a direct protein–protein interaction between host PrP^C and inoculated PrP^{Sc}, with PrP^{Sc} acting to promote further conversion of PrP^C to PrP^{Sc} in an autocatalytic process which proceeds most efficiently when the interacting proteins are of identical primary structure^{4–7}.

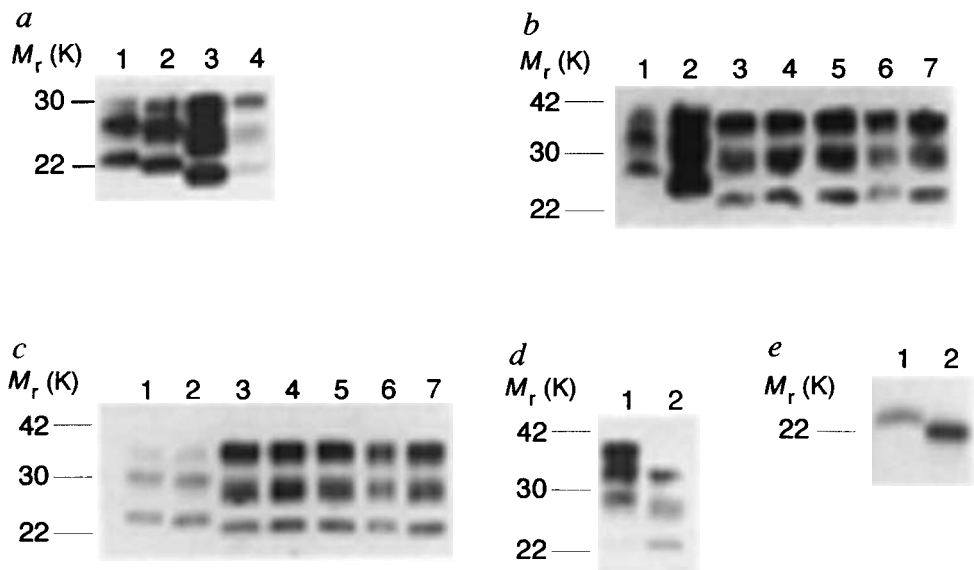
In addition to the unique biology of these diseases, interest in them has been intensified because of the epidemic of the prion disease bovine spongiform encephalopathy (BSE)⁸ in the UK, and now in other countries, and the possibility that this may represent a significant threat to public health through ingestion of BSE-infected tissues. BSE is known to have caused prion disease in a number of other species, including domestic cats (feline spongiform encephalopathy) and captive exotic ungulates (nyala and kudu), presumably as a result of ingestion of BSE-contaminated feed⁹. The pathogenicity of bovine prions for humans is unknown, although the results of exposing transgenic mice expressing human prion protein, which lack a species barrier to human prions, indicate that induction of human prion production by bovine prions is inefficient¹⁰.

Although many converging lines of evidence support the 'protein only' hypothesis for prion propagation¹, the existence of several distinct isolates or 'strains' of agent that can be stably passaged in inbred mice of the same prion protein genotype has yet to be explained satisfactorily within this model. Strains can be distinguished by their different incubation periods and patterns of neuropathology when passaged in mice¹¹. A number of distinct strains of natural sheep scrapie are recognized, although BSE seems to be caused by a single strain of agent⁹.

Support for the idea that strain specificity is encoded by PrP alone is provided by study of two distinct strains of transmissible mink encephalopathy prions, designated hyper (HY) and drowsy (DY), which can be serially propagated in hamsters¹². These strains can be distinguished by differing physicochemical properties of the accumulated PrP^{Sc} in the brains of affected hamsters¹³. After limited proteolysis, strain-specific migration patterns of PrP^{Sc} on polyacrylamide gels can be seen. DY PrP^{Sc} seems to be more protease-sensitive than HY PrP^{Sc}, producing a different banding pattern of PrP^{Sc} on western blots after proteinase K treatment. This relates to different amino-terminal ends of HY and DY PrP^{Sc} after protease treatment and implies differing conformations of HY and DY PrP^{Sc} (ref. 14). Furthermore, the demonstration that these strain-specific physicochemical properties can be maintained during *in vitro* production of protease-resistant PrP, when PrP^C is mixed with HY or DY hamster PrP^{Sc}, further supports the concept that prion strains involve different PrP conformers¹⁵.

The human prion diseases occur in inherited, acquired and sporadic forms. About 15% are inherited, and are associated with coding mutations in the prion-protein gene (*PRNP*)¹⁶. Acquired prion diseases include kuru and iatrogenic Creutzfeldt–Jakob disease (CJD). Recognized iatrogenic routes of transmission are treatment with human cadaveric pituitary-derived growth hormone or gonadotrophin, dura mater or corneal grafting, and the use of inadequately sterilized neurosurgical instruments¹⁷. However, the majority of human prion disease occurs as sporadic CJD, in which pathogenic *PRNP* mutations and a history of iatrogenic exposure are absent. The majority of sporadic CJD cases are homozygous at polymorphic residue 129, a common protein polymorphism in human PrP that is known to be important in genetic susceptibility to human prion diseases^{6,18–20}. Recently, the occurrence of a new form of human prion disease was reported in the United Kingdom, affecting unusually young people and having a highly consistent and unique clinicopathological pattern²¹. To date, all patients studied are homozygotes (for methionine) at polymorphic residue 129 of PrP and no coding mutations are present²². None has a history of iatrogenic exposure to human prions. This may indicate the arrival of a new risk factor for CJD in the United Kingdom, with dietary exposure to specified bovine offals, before their statutory exclusion from the human diet in 1989, seeming to be the most likely candidate. It is not known

FIG. 1 Western blots of proteinase K-treated brain homogenates from patients with sporadic and new variant CJD using anti-PrP monoclonal antibody 3F4. Numbers adjacent to horizontal bars indicate positions of M_r markers. a, Lane 1, type-1 sporadic CJD, *PRNP* genotype MM; lane 2, type-2 sporadic CJD, *PRNP* genotype MM; lane 3, type-3 iatrogenic CJD, *PRNP* genotype VV; lane 4, new variant CJD, *PRNP* genotype MM, 'type-4' banding pattern. b, Lanes 1 and 2, type-1 and type-2 sporadic CJD, respectively (both with *PRNP* MM genotype; lanes 3–7, new variant CJD cases (all *PRNP* genotype MM). c, Lane 1, type-2 sporadic CJD, *PRNP* genotype MV; lane 2, type-2 sporadic CJD, *PRNP* genotype VV; lanes 3–7, new variant CJD (all *PRNP* genotype MM). d, New variant CJD before (1) and after (2) treatment with proteinase K. e, Western blot of deglycosylated PrP. Lane 1, type-2 sporadic CJD, *PRNP* genotype MM; lane 2, new variant CJD, *PRNP* genotype MM.



how many strains of human prions cause CJD; only two distinct patterns of protease-resistant PrP have been reported to date, associated with different clinicopathological types of sporadic CJD²³.

We have investigated a wide range of cases of human prion disease to identify patterns of protease-resistant PrP that might indicate different, naturally occurring, prion strain types. We then studied 'new variant' CJD to determine whether it represents a distinct strain type that can be differentiated by molecular criteria from other forms of CJD. We demonstrate that sporadic and iatrogenic CJD is associated with three distinct patterns of protease-resistant PrP on western blots. Types 1 and 2, as previously described, are seen in sporadic CJD, and also in some iatrogenic CJD cases. A third type is seen in acquired prion diseases that arise from a peripheral route of exposure to prions. New variant CJD is associated with a unique and highly consistent appearance of protease-resistant PrP on western blots, involving a characteristic pattern of glycosylation. Whereas transmission of CJD to inbred mice produces a pattern characteristic of the inoculated CJD, transmission of BSE produces a glycoform ratio pattern closely similar to new variant CJD. Similarly, experimental BSE in macaques and naturally acquired BSE in domestic cats show an indistinguishable glycoform pattern to experimental murine BSE and new variant CJD. Transmission of types 1, 2 and 3 CJD to transgenic mice expressing human PrP reveals persistence or conversion of strain type dependent on *PRNP* codon-129 genotype, providing supportive evidence for the 'protein only' hypothesis of infectiousness and indicating that strain variation might be encoded by a combination of PrP conformation and glycosylation.

Sporadic and acquired CJD

Typically, three bands are seen on western blots of protease-resistant PrP from CJD cases. The two larger-molecular-mass bands represent the main glycosylated forms of PrP and the smaller band represents unglycosylated PrP²³ (Fig. 1). Two distinct patterns have been described in sporadic CJD: a type-1 pattern seen in the majority of CJD cases, with homozygosity for methionine (MM) at polymorphic residue 129 of PrP, and a type-2 pattern seen in a minority of MM cases and in all methionine/

valine heterozygotes (MV) and valine homozygotes (VV)²³. We used western blotting to analyse protease-resistant prion proteins for a total of 26 neuropathologically confirmed sporadic CJD cases representing all three *PRNP* codon-129 genotypes. Cases that pre-dated and cases that were contemporary to the BSE epidemic were included (Table 1). We confirmed the finding²³ that in sporadic CJD, MM cases had two distinct banding patterns (designated types 1 and 2) (Fig. 1a, b and Table 1), whereas VV and MV cases all showed the type-2 banding pattern²³ (Fig. 1c and Table 1). In our sample of sporadic CJD cases, we found type-2 MM to be more frequent than type-1 MM (Table 1). In addition, differences were previously noted between the proportion of protease-resistant PrP in each of the three bands between the type-1 and type-2 sporadic CJD patients²³. In our larger series, a statistically significant difference was seen between type-1 and type-2 cases with respect to the proportion of the low-molecular-mass glycoform, but not with respect to the other two bands (Fig. 4 legend).

We then studied a range of iatrogenic CJD cases, including patients who had received human pituitary-derived growth hormone, with all three *PRNP* codon-129 genotypes, a human

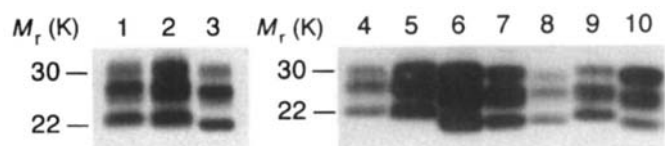


FIG. 2 Western blots of proteinase K-treated brain homogenates from patients with iatrogenic CJD, using anti-PrP monoclonal antibody 3F4. Lanes: 1, Sporadic CJD (*PRNP* genotype MM) type 1; 2, iatrogenic (growth-hormone related) CJD (*PRNP* genotype MM) type 1; 3 and 4, sporadic CJD (*PRNP* genotype MM) type 2; 5, iatrogenic (dura mater-related) CJD (*PRNP* genotype MM) type 2; 6 and 7, iatrogenic (growth hormone-related) CJD (*PRNP* genotype VV) type 3; 8, iatrogenic (growth hormone-related) CJD (*PRNP* genotype MV) type 3; 9, sporadic CJD (*PRNP* genotype MV) type 2; 10, new variant CJD (*PRNP* genotype MM) type 4.

TABLE 1 *PRNP* genotypes and banding type of protease-resistant PrP in sporadic, iatrogenic and new variant CJD

Classification	Codon-129 genotype	Type 1	Type 2	Type 3	Type 4	Total
Sporadic	MM	5	13	0	0	18
Sporadic	MV	0	4	0	0	4
Sporadic	VV	0	4	0	0	4
Iatrogenic (growth hormone)	MM	1	0	0	0	1
Iatrogenic (growth hormone)	MV	0	0	1	0	1
Iatrogenic (growth hormone)	VV	0	0	3	0	3
Iatrogenic (gonadotrophin)	VV	0	0	1	0	1
Iatrogenic (dura mater)	MM	0	1	0	0	1
New variant	MM	0	0	0	10	10

MM, methionine-homozygous genotype at *PRNP* codon 129; MV and VV, methionine/valine heterozygotes and valine homozygotes, respectively.

pituitary-derived gonadotrophin patient, and a patient who developed CJD after a dura mater graft. A third distinct banding pattern of protease-resistant PrP was detected in all of the pituitary hormone-related cases which were of *PRNP* codon-129 genotype MV or VV (Fig. 2). In this type-3 pattern, all three bands are shifted, consistent with a decrease in relative molecular mass (M_r) of roughly 2,000 to 3,000 (2K–3K) of the protease-resistant PrP detected as compared with type-2 sporadic CJD. There were no significant differences in glycoform ratios between type-1 and type-3 or between type-2 and type-3 CJD in this sample (Fig. 4 legend). The single MM growth-hormone case had a banding pattern indistinguishable on western blot analysis from type-1 sporadic CJD cases (Fig. 2). The dura mater-related case had a banding pattern indistinguishable from type-2 sporadic CJD (Fig. 2). The possibility that there may be further heterogeneity within these individual PrP^{Sc} types is under investigation.

Transmission studies to mice

We are studying the transmission characteristics of CJD in transgenic mice that express human PrP but not murine PrP (designated HuPrP^{+/+} *Pm-p*^{0/0} as they are homozygous for the human PrP transgene array)¹⁰. These mice lack a species barrier to human prions, and most inoculated mice succumb to prion disease with consistently short incubation periods^{10,24}, usually in the region of 180–220 days (data not shown). This is markedly different from studies with non-transgenic mice using the same inocula, in which transmissions are infrequent and when they do occur they are associated with prolonged and variable incubation periods.

Transmission of type-2 CJD (of all three codon-129 genotypes) or type-3 CJD (which were all of genotype MV or VV) resulted in production of protease-resistant human PrP in the mice with an identical banding pattern to the primary inoculum (type 2 or 3, respectively) (Fig. 3). The HuPrP^{+/+} *Pm-p*^{0/0} mice used encode

valine at residue 129 (ref. 25). However, transmission of type-1 CJD (which are all of genotype MM) resulted consistently in a type-2 banding pattern of human protease-resistant PrP produced in the mice (Fig. 3). The proportion of different glycoforms on western blots of brain homogenates from these mice was indistinguishable from that of the human cases themselves (data not shown).

New variant CJD

Proteinase K treatment of PrP^{Sc} from new variant CJD revealed the characteristic band shift seen after digestion to the protease-resistant fragment, confirming complete digestion of the protease-sensitive N-terminal region of PrP^{Sc} in the conditions used (Fig. 1d). All patients studied with new variant CJD were homozygotes for methionine at polymorphic residue 129 (ref. 22). No known or new coding mutations of *PRNP* were seen in the new variant CJD cases or the sporadic CJD cases that were sequenced. Western blot analysis of these ten new variant CJD cases revealed a consistent and distinct pattern of protease-resistant PrP forms, which could be clearly differentiated by band sizes from type-1 and type-2 sporadic CJD cases (Fig. 1a–c), and from type-3 CJD by a striking and distinctive pattern of band intensities which differed from all three CJD types (Fig. 4). Deglycosylation with PNGaseF resulted in a single band, indicating that there might be a consistent proteolytic cleavage site irrespective of glycosylation state (Fig. 1e) and which differs from that seen in sporadic CJD. The high- M_r glycoform was the most abundant, with relatively little unglycosylated PrP when compared with type-1, type-2 and type-3 CJD. These differences in band intensity from types 1–3 CJD were all highly statistically significant (Fig. 4 legend). A scattergram of the relative proportions of the high- M_r and low- M_r glycoforms reveals two non-overlapping populations of cases: new variant CJD has a distinctive pattern that differs markedly from

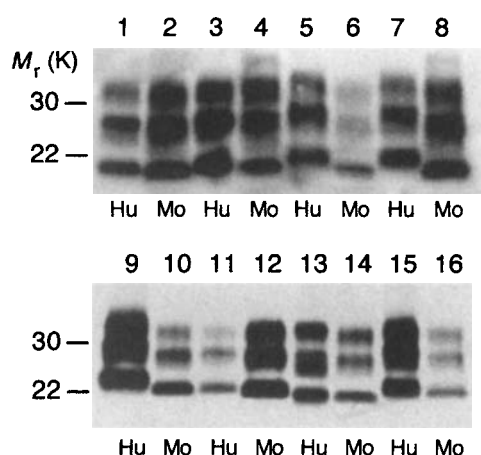


FIG. 3 Transmission of CJD to transgenic mice expressing only human PrP. Western blots of primary human inocula and mouse brain homogenates after treatment with proteinase K using the anti-PrP monoclonal antibody 3F4. Hu, human inoculum; Mo, indicates transgenic mice that developed disease after inoculation with this human case. Lanes 1 and 2, sporadic CJD (MV) type 2 → mouse type 2; lanes 3 and 4, sporadic CJD (MV) type 2 → mouse type 2; lanes 5 and 6, iatrogenic (growth hormone-related) CJD (MM) type 1 → mouse type 2; lanes 7 and 8, sporadic CJD (MM) type 1 → mouse type 2; lanes 9 and 10, sporadic CJD (MM) type 1 → mouse type 2; lanes 11 and 12, sporadic CJD (VV) type 2 → mouse type 2; lanes 13 and 14, iatrogenic (gonadotrophin related) CJD (VV) type 3 → mouse type 3; lanes 15 and 16, iatrogenic (dura mater-related) CJD type 2 → mouse type 2.

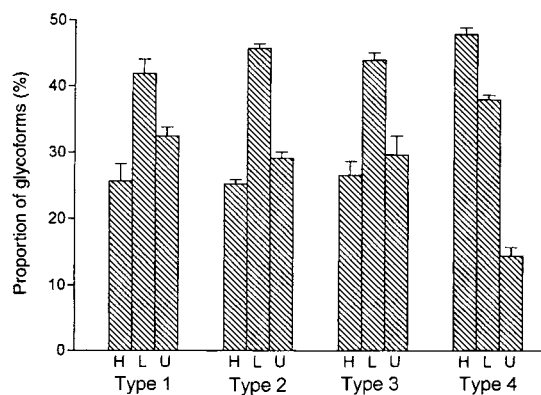


FIG. 4 Proportions of different PrP glycoforms in type-1, type-2, type-3 and type 4 (new variant) CJD. Percentage of each form is given as mean $\pm$ s.e.m. of the three banding types. H, high-molecular-mass glycoform; L, lower-molecular-mass glycoform; U, unglycosylated. Sample sizes were: type 1, $n = 6$; type 2, $n = 18$; type 3, $n = 4$; new variant CJD (type 4), $n = 9$. Differences between type 1 and type 2 were significant with respect to the low- M_r glycoform ($P < 0.05$) but not with the high- M_r glycoform or unglycosylated form. There were no significant differences between types 1 and 3 and between types 2 and 3. All three bands were highly significantly different between type 4 and type 1, type 2 and type 3 (high- M_r glycoform, each case $P < 0.0001$). In all cases unpaired two-tailed t-tests were performed.

all previously recognized types of sporadic and iatrogenic CJD (Fig. 5a).

Comparison with BSE

As the band intensities of protease-resistant PrP in new variant CJD differed markedly from sporadic and iatrogenic CJD, we investigated whether this distinctive glycoform pattern was also seen in naturally or experimentally transmitted BSE. First, we compared transmissions of BSE and CJD to the same inbred mice. Comparative transmission data in transgenic mice was not available as BSE has not transmitted, to date, to HuPrP^{+/+} *Pm-p*^{0/0} mice (>500 days post-inoculation). The glycoform ratios seen in CJD transmissions to wild-type FVB mice were indistinguishable from those of the three types of CJD (Fig. 5b). However, BSE transmission into both wild-type FVB and C57BL mice resulted in ratios that were closely similar to those of new variant CJD (Figs 5b, 6). Similarly, band sizes of protease-resistant PrP seen on transmission of BSE to wild-type mice were shifted to a lower M_r as compared to type 2 CJD transmission (data not shown). We then studied naturally transmitted BSE in the domestic cat (feline spongiform encephalopathy²⁶) and experimental BSE in a macaque²⁷. These cases also closely resembled new variant CJD and experimental murine BSE (Figs 7a, 5b). BSE itself was not detectable on western blots using the 3F4 monoclonal or R073 polyclonal antibody. However, a PrP^{Sc} signal was detected from

homogenates of brainstem from naturally infected BSE using a rabbit antibody to a synthetic human PrP peptide (95–108) (provided by B. Ghetti) and the pattern of the glycoforms (51.2% high M_r , 33.9% low M_r , 14.9% unglycosylated) was closely similar to transmitted BSE and new variant CJD. This antibody also detected PrP^{Sc} from domestic cat, macaque and humans, producing similar results to R073 and 3F4 antisera (data not shown).

Discussion

Sporadic and iatrogenic CJD seems to be related to the production of three distinct types of human PrP^{Sc} which can be differentiated on western blots after proteolytic cleavage by their differing band sizes. Types 1 and 2 are associated with different clinicopathological phenotypes of sporadic CJD²³, and type 3 is seen in cases of iatrogenic CJD where exposure to prions has been via a peripheral route (intramuscular injection of human cadaveric pituitary-derived hormones) rather than by a direct central nervous system (CNS) route (dura mater grafting). It is well recognized that such peripherally acquired cases have a distinct phenotype, presenting with cerebellar ataxia and psychiatric disturbance rather than as a dementing illness²⁸. Iatrogenic CJD resulting from CNS exposure typically resembles classical sporadic CJD²⁸. New variant CJD, while it has PrP^{Sc} band sizes similar to type-3 CJD, can be clearly distinguished from all three types of

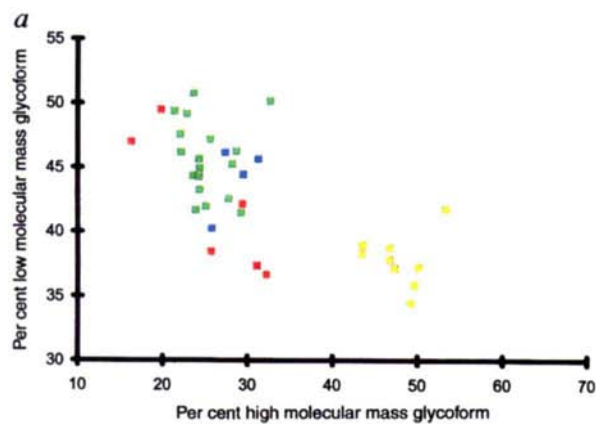
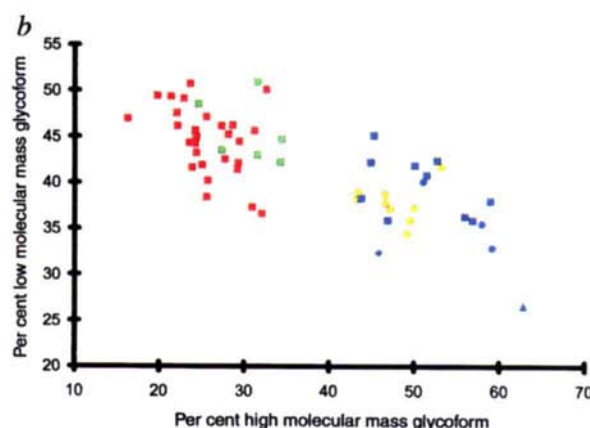


FIG. 5 Scattergraph of proportions of protease-resistant PrP in the high-molecular-mass and low-molecular-mass glycoform in individual human cases and animals with transmitted CJD or naturally or experimentally transmitted BSE. a, Comparison of sporadic, iatrogenic and new variant CJD. Type-1 CJD is represented by red squares, type 2 by green squares, type 3 by blue squares and type 4 (new variant CJD) by yellow squares. b, Comparison of CJD types with naturally and experimentally transmitted



BSE. All sporadic and iatrogenic CJD (types 1–3) are represented by red squares, and new variant cases are represented by yellow circles. CJD transmissions to FVB mice are denoted by green squares, BSE transmissions to FVB and C57BL/6 mice by blue squares and blue circles, respectively. Naturally transmitted BSE in a domestic cat is denoted by a blue triangle and experimental BSE in macaque by a blue diamond shape.

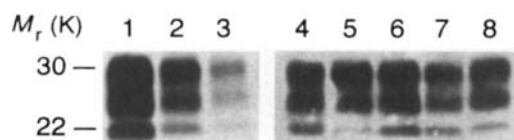


FIG. 6 Transmission of BSE to wild-type C57BL/6 and FVB mice. Western blot of brain homogenates after pre-treatment with proteinase K using anti-PrP antibody R073. Lanes 1–3, C57BL/6 mice; lanes 4–8, FVB mice.

CJD by a characteristic pattern of band intensities. This distinctive molecular marker, which clearly differentiates new variant CJD from sporadic CJD, serves to support the proposal, on the basis of comparative clinicopathological studies and epidemiological surveillance²¹, that new variant CJD is a distinct and new subtype of prion disease, related to a previously unrecognized prion strain. The spontaneous occurrence of a novel human PrP^{Sc} type, that is the same in twelve individuals in the United Kingdom, over the last two years, seems extraordinarily unlikely as an explanation for 'new variant' CJD. The alternative conclusion is that these cases have arisen from a common source of exposure to a new prion strain, and the lack of any history of common iatrogenic exposure indicates that this is probably a new animal strain. That the glycoform 'signature' of new variant CJD is seen in BSE itself, in experimental murine BSE (whereas CJD transmission to these types of mice produces the CJD 'signature') and in naturally transmitted BSE in domestic cat and experimental BSE in macaque, is consistent with the hypothesis that new variant CJD results from BSE transmission to humans. Transmission studies of new variant CJD in HuPrP^{+/+} *Pm-p*^{0/0} mice are in progress; it will be of interest to compare incubation periods and patterns of neuropathology with other CJD transmissions (which are also currently under investigation). PrP^{Sc} typing will be of immediate application in wider epidemiological studies of CJD. It is possible that BSE could produce other clinicopathological phenotypes in humans, particularly in different age groups and different ethnic populations, that are not recognized as new variant CJD. Furthermore, this method may also allow typing of various animals to see if BSE has also transmitted naturally to these species. There is particular concern that BSE may have transmitted to, and be surviving in, the sheep population²⁹. Typing of known scrapie strains which pre-dated the BSE epidemic, and recent isolates, will be important in this regard.

This molecular marker can already be used in differential diagnosis of new variant CJD. New variant CJD is atypical both in its clinical features and electroencephalogram, such that diagnosis is dependent on neuropathology, either at autopsy or in some cases on brain biopsy. However, although the brain biopsy may demonstrate spongiform encephalopathy and PrP immunoreactivity adequate for a diagnosis of CJD, the characteristic neuropathological features necessary for a diagnosis of new variant CJD may not be present in the biopsy sample²¹. As PrP is expressed in the lymphoreticular system, and prion replication occurs in spleen and in other lymphoreticular tissues³⁰, it may be possible to detect this molecular marker of new variant CJD in tonsil³¹ or lymph-node biopsy and thereby avoid brain biopsy.

The aetiology of sporadic CJD remains unclear but may involve somatic *PRNP* mutations or spontaneous conversion of PrP^C to PrP^{Sc} as a rare stochastic event. Sporadic CJD is associated with type-1 or type-2 PrP^{Sc}. Type 1 is always associated with genotype MM, type 2 with all genotypes (MM, MV or VV). Type 3 is seen in iatrogenic CJD of genotype MV or VV. Only human PrP M129 seems to form type-1 PrP^{Sc}, whereas either human PrP M129 or human PrP V129 can produce type-2 PrP^{Sc}. As type-3 PrP^{Sc} is only seen in MV or VV individuals, it is possible that only human PrP

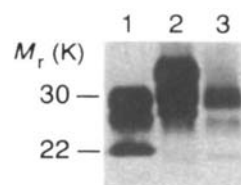


FIG. 7 Western blot of brain homogenates from a BSE-inoculated macaque (lane 1) using antibody 3F4 after pre-treatment with proteinase K and of a domestic cat with feline spongiform encephalopathy (lanes 2 and 3, before and after treatment with proteinase K, respectively).

V129 can form this type. As type-3 PrP^{Sc} is seen in peripherally acquired iatrogenic CJD cases, it is possible that this strain is selected in or preferentially produced by the lymphoreticular system, where prion replication first occurs in the experimentally transmitted disease in mice³². If such a PrP^{Sc} type were formed preferentially from human PrP V129, this could explain the excess of the *PRNP* codon-129 VV genotype among pituitary-hormone related CJD cases^{19,20,33}. Further studies will be required to determine whether the type-3 pattern is a consistent marker of peripheral, as opposed to central, prion exposure or sporadic CJD. It is of note, however, that similar band sizes are seen in the type 4 (new variant CJD) pattern (which are of *PRNP* genotype MM), which seems to have arisen by peripheral (presumably dietary) exposure to bovine prions. The formation of particular PrP^{Sc} types seems to be constrained by host *PRNP* codon-129 genotype. This finding is supported by the observation that type-1 PrP^{Sc} converts to type 2 on passage in transgenic mice expressing the human PrP gene encoding valine at residue 129, whereas types 2 and 3 remain unchanged on such passage.

The different types of human PrP^{Sc} are seen in association with distinct clinicopathological phenotypes of CJD, and can be maintained on passage in mice, which indicates that these represent distinct human prion strains. The finding that strains appear to involve different post-translational modifications of PrP, which persist or (when PrP genotypes are mismatched) can be predictably converted between discrete strains on passage in mice, is consistent with a protein-only model of prion propagation in which strains are encoded by post-translational modification of PrP itself without the need for a nucleic acid or other cofactor. The bands seen on western analysis of PrP following proteolytic cleavage represent diglycosylated, monoglycosylated (at either of the two N-linked glycosylation sites³⁴) and unglycosylated PrP, and two separate features of these bands, shifts in mobility and differences in relative intensities, seem to be associated with strain type. The mobility shifts after cleavage, seen in all three bands, imply different PrP conformations (which may include differing states of assembly). The differences in glycoform ratios could indicate preferential conversion of particular glycoforms into particular conformational states. It has been argued that differing PrP glycosylation in different brain regions could provide a mechanism for the targeting of neuropathology seen with different strain types, as prions may replicate most efficiently in cell populations expressing a similarly glycosylated PrP on the cell surface. Both PrP conformation and glycosylation may contribute to strain type but further studies will be required to investigate whether these two post-translational modifications of PrP can contribute to strain type independently, or are coupled phenomena. □

Methods

Selection and molecular genetic analysis of patients. Twenty-six neuropathologically confirmed sporadic CJD cases including all three codon-129 genotypes, seven neuropathologically confirmed iatrogenic CJD cases and ten neuropathologically confirmed new variant CJD cases referred to the National

CJD Surveillance Unit or the Prion Disease Group, from which frozen brain tissue was available, were studied. Brain samples used were from the cerebral cortex, usually frontal cortex. DNA was extracted from blood or brain tissue and analysed for the presence of known or new coding mutations in the prion protein gene (*PRNP*) and to determine codon-129 genotype. In 8 of 10 new variant CJD and the majority of sporadic and iatrogenic CJD patients, the complete *PRNP* open reading frame was amplified by polymerase chain reaction (PCR) using oligonucleotide primers chosen so as not to overlay an intron polymorphism 5' to the open reading frame, which can lead to non-amplification of certain alleles, as described previously³⁵. The PCR product was size-fractionated in agarose gels to exclude insertional or deletional mutations, and were then sequenced on both DNA strands using an ABI 373 or 377 automated DNA sequencer (Applied Biosystems).

Western blot analysis. Between 10 and 20 mg of brain tissue was homogenized in lysis buffer (0.5% NP-40, 0.5% sodium deoxycholate in phosphate buffered saline) by serial passage through needles of decreasing diameter. The homogenate was cleared by centrifugation at 2,000 r.p.m. for 5 min. Proteinase K (BDH) was added to a final concentration of 50 µg ml⁻¹ and the samples incubated at 37 °C for 1 h. The reaction was terminated by the addition of Pefabloc (Boehringer) to 1 mM. The samples were mixed with twice the volume of SDS loading buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, pH 6.8) and boiled for 10 min. They were then centrifuged at 14,000 r.p.m. in a microfuge for 5 min before electrophoresis. Between 1 and 10 µl of sample was electrophoresed on 16% Tris-glycine gels (Novex)³⁶. The gels were then electroblotted onto PVDF membrane (Millipore) using either a tank or semi-dry blotting system³⁷. The membranes were blocked in 5% BLOTTO (5% non-fat milk powder in PBS with 0.05% Tween 20) for 1 h at room temperature. No single antibody detects all species studied efficiently. After washing in PBST (PBS with 0.05% Tween 20), the membranes were incubated with anti-PrP monoclonal antibody 3F4 (ref. 38) (diluted 1:5,000 in PBST), rabbit polyclonal antibody R073 (ref. 39) (diluted 1:10,000 in PBST), or rabbit anti-human PrP peptide (95–108) (diluted 1:20,000) (from B. Ghetti) for between 1 h and overnight. After washing, the membrane was incubated with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Sigma) or goat anti-rabbit antibody (Sigma) at a dilution of 1:10,000 in PBST for 1 h. The

membranes were washed again in PBST and developed using a chemiluminescent substrate (ECL; Amersham) using Biomax MR film (Kodak).

Deglycosylation of prion proteins. A portion (25 µl) of 10% brain homogenate, pre-treated with proteinase K, was denatured in 0.5% SDS, 1% β-mercaptoethanol for 10 min at 100 °C. NP-40 was added to 1% and the proteins were incubated in 500 units PNGaseF (New England Biolabs), using the proprietary buffer, at 37 °C for 2 h. After digestion, proteins were precipitated with 4 volumes of methanol and resuspended in SDS loading buffer and western blotted.

Quantitation of PrP glycoform ratios. Blots were scanned on a Hoefer scanning densitometer (model GS300) and the relative amounts of the different glycoforms were obtained by computerized integration of peaks representing each of the three distinct bands. Scanning was done on exposures within the linear range of the photographic film.

Transmission studies in transgenic and non-transgenic mice. Strict biosafety methods were followed. Transgenic mice expressing human PrP were bred and maintained in an animal microbiological containment level I facility and moved to a containment level II facility where intracerebral inoculation was performed in a class I microbiological safety cabinet. Preparation of inocula and removal of tissues were done in a microbiological containment level III facility. All mice were examined twice weekly for the development of clinical signs of scrapie. At onset of signs, mice were examined daily. Mice were killed if exhibiting any signs of distress. Criteria for clinical diagnosis of scrapie in mice were as described previously⁴⁰. Transgenic lines expressing human PrP but not murine PrP were established by breeding mice transgenic for human PrP (designated Tg152, produced as described previously²⁵) with mice homozygous for PrP null alleles⁴¹. All mice were genotyped to confirm the presence of the HuPrP transgene or PrP null alleles by PCR with genomic DNA obtained by tail biopsy, as described⁴². Tg152 mice have expression levels of human PrP of 200% of that seen in normal human brain⁴². Mice were anaesthetised with halothane/O₂ and intracerebrally inoculated into the right parietal lobe with 30 µl of a 1% brain homogenate in phosphate-buffered saline.

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A transcriptional partner for MAD proteins in TGF- β signalling

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The transforming-growth-factor- β (TGF- β) superfamily is critical for establishing mesoderm during early embryogenesis in *Xenopus*. The transcriptional activation of *Mix.2*, an immediate-early response gene specific to activin-like members of the TGF- β superfamily, is associated with the rapid appearance of a site-specific DNA-binding activity that recognizes a fifty-base-pair regulatory element known as ARE in the *Mix.2* promoter. Cloning of the site-specific DNA-binding component of this activity revealed it to be a new winged-helix transcription factor and a direct target for signalling by the TGF- β superfamily. X_{MAD}2, a recently identified TGF- β signal transducer, forms a complex with the transcription factor in an activin-dependent fashion to generate an activated ARE-binding complex. A model is proposed to explain how TGF- β superfamily signals might regulate the expression of specific genes in the early embryo.

THE TGF- β superfamily of cytokines, including TGF- β , activin and the bone-morphogenic proteins (BMPs), elicits a broad range of cellular responses, including the regulation of cell division, survival, differentiation and specification of developmental fate^{1,2}. In the early frog embryo, this family plays a central role in the specification and patterning of several tissues³⁻⁵. Activin, Vg-1, and TGF- β all induce a full range of dorsal and ventral mesodermal markers in early embryonic tissue⁶⁻⁹, whereas other TGF- β superfamily members specify axial pattern or epidermal as opposed to neural tissue¹⁰⁻¹⁶. Almost all the critical patterning events in early *Xenopus* embryogenesis appear to involve some members of the TGF- β superfamily, and overexpression in the embryo of a truncated type-II activin receptor that inhibits signalling by TGF- β superfamily ligands can almost completely suppress mesoderm formation and axial patterning^{17,18}.

Many transcriptional responses of embryonic blastomeres to TGF- β superfamily signals are necessary or sufficient to specify or pattern mesoderm. Several responsive genes show immediate-early responses: they are induced even when translation is inhibited by cycloheximide. Two such genes, *Mix.1* and *Mix.2*, respond to several TGF- β superfamily signals, including activin, Vg-1, TGF- β , and BMP4 (refs 19-21), but not to non-TGF- β mesoderm inducers or axial modifiers. *Mix.1* and *Mix.2*, closely related homeobox genes with identical patterns of expression and induction, are expressed in the prospective mesoderm and endoderm just after the mid-blastula transition^{19,22}. A 290-base-pair (bp) element from the promoter of the *Mix.2* gene confers a cycloheximide-insensitive activin response, thus defining this region as a target for immediate-early transcriptional regulation in response to embryonic TGF- β superfamily signals²².

How do transcription factors mediate immediate-early regulation of the *Mix.2* promoter by the TGF- β superfamily? Using an electrophoretic mobility-shift assay (EMSA) for embryonic proteins that bind to the *Mix.2* promoter elements²⁰, we identified a factor induced in embryonic blastomeres after 5-30 minutes of activin stimulation that binds specifically to a 50-bp *Mix.2* promoter element. This activin-responsive factor (ARF) is inducible by Vg-1 and TGF- β as well as by activin, and can be induced in the presence of cycloheximide, indicating that it is an immediate-early response peculiar to TGF- β superfamily ligands²⁰. The activin-responsive element (ARE) to which this factor binds is necessary and sufficient for most of the activin responsiveness of *Mix.2*; this element therefore appears to be a critical target for regulation by activin/Vg-1/TGF- β signalling²⁰.

We now report the cloning of a major DNA-binding component of ARF. This novel winged-helix transcription factor is present maternally in early embryos. We show that the ARF complex contains X_{MAD}2, a *Xenopus* homologue of the *Drosophila* Mad gene which is linked to activin signalling²³. These observations provide a direct physical connection between a proposed TGF- β superfamily signal transducer and a transcription factor with DNA-binding specificity for a TGF- β -superfamily-responsive promoter, suggesting a simple model: cytoplasmic MAD activation leads to the specific activation of a TGF- β -superfamily-responsive gene in the early embryo.

Cloning of an ARE-binding protein

To identify components of ARF, we screened for DNA-binding proteins that recognize ARE with a specificity similar to the DNA-binding component(s) of ARF. By visual inspection, we identified 6-bp and 7-bp repeated sequences within the ARE (Fig. 1a). We synthesized 50-bp ARE oligonucleotides containing point mutations in both copies of each of the repeats and tested their ability to bind to ARF; mutations in the 7-bp repeats do not significantly reduce ARE binding (not shown), whereas point mutations in the 6-bp repeats eliminate ARE binding (Fig. 1a, b).

A *Xenopus* oocyte complementary DNA library in the pGAD10 fusion vector (Clontech), which provides an amino-terminal GAL4 transcriptional activation domain, was transformed into a yeast strain bearing a trimerized ARE sequence upstream of *HIS3* and *lacZ* reporter genes. DNA from positive colonies was then transformed into yeast containing a wild-type or mutant ARE upstream of *lacZ*. Twenty-three clones, representing 11 distinct inserts, were identified that bound in the wild-type ARE strain and did not bind in the mutant ARE strain. Of these, three contained open reading frames that were readily identifiable by sequencing from the GAL4 fusion side of the insert. Two of these, clones 1H208 and 1H280, contained overlapping open reading frames apparently from the same gene, being identical in amino-acid sequence and 99.5% similar at the nucleotide level over their 600 nucleotides of overlap. Clone 1H208 contains a 3.2-kilobase (kb) insert encoding a predicted protein in frame with the GAL4 fusion domain of relative molecular mass (M_r) 51K, but which presumably lacks its normal N terminus, which in this clone is replaced by the GAL4 activation domain. Clone 1H280 contains a 1-kb insert, the open reading frame of which is not in frame with the GAL4 activation domain, but rather begins 123 base pairs 3' to cloning site (this 123-bp region contained stop codons in all frames). The

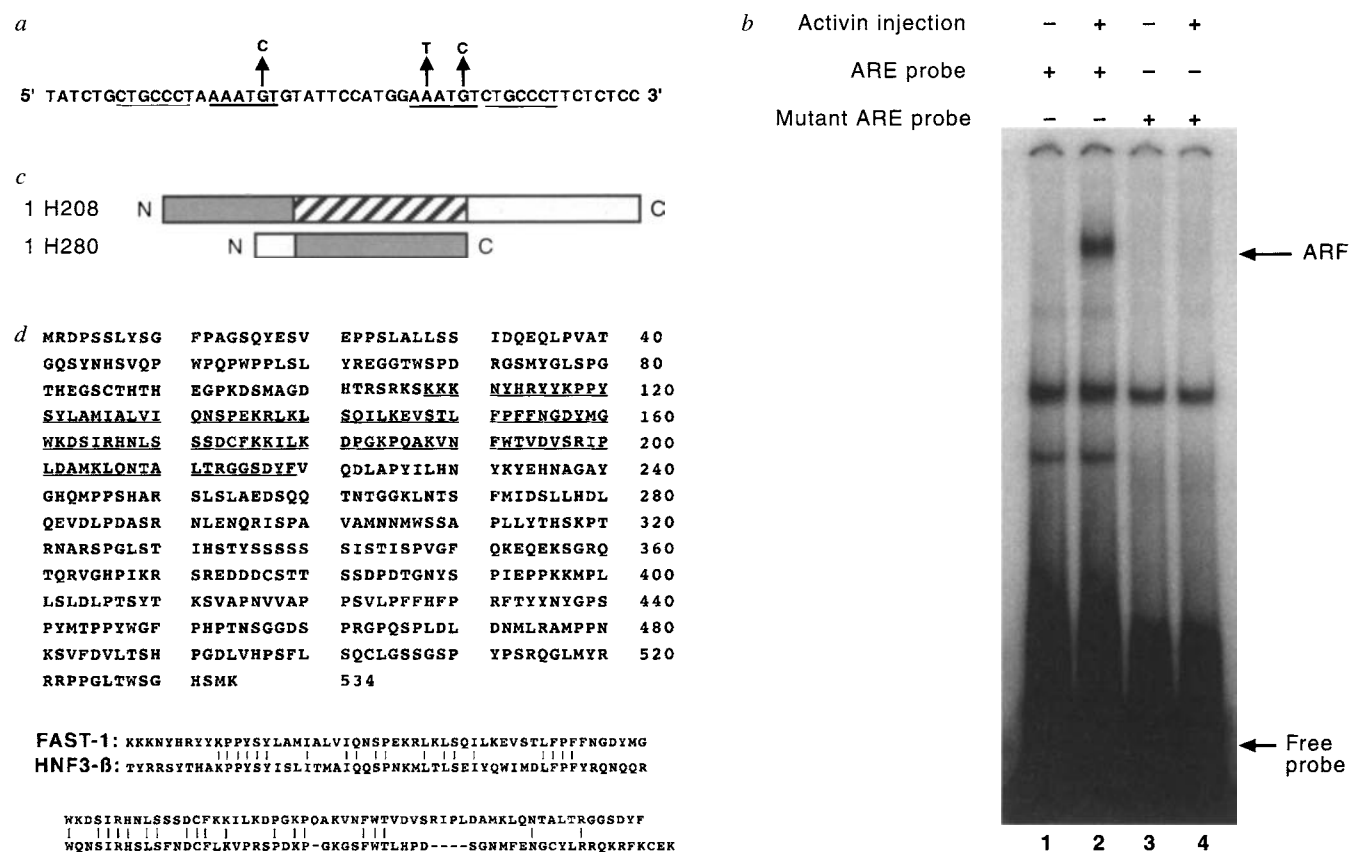


FIG. 1 Cloning of an ARE-specific DNA-binding protein. **a**, Position of repeat elements in ARE and mutations in the 6-bp repeat. Sequence of the ARE from the *Mix.2* promoter is shown. The 6-bp direct repeats are indicated by bold underlining; light underline indicates 7-bp repeats. Arrows indicate sites of directed mutation of the 6-bp repeats. **b**, Binding of ARF to wild-type or mutated ARE oligonucleotide probe in EMSA. **c**, Predicted proteins from overlapping sequence of clones encoding ARE-specific DNA-binding activity. Shaded box region indicates in-frame GAL4 activator domain

sequence fused to insert ORF; striped box indicates regions of identical, overlapping insert of clones 1H208 and 1H280; open box indicates non-overlapping insert sequence in clones 1H208 and 1H280. **d**, Top: predicted protein sequence of FAST-1. The predicted winged-helix domain is underlined. Bottom: comparison of the FAST-1 winged-helix domain to the rat HNF-3 β winged-helix domain is shown; lines indicate identical amino acids between the aligned sequences. The GENBANK accession number for the sequence reported here is U70980.

near-identity of nucleotide sequence between 1H280 and 1H208, up to the junction of each with the pGAD10 vector, indicated that they represent different regions of the same gene; 1H280 containing the amino terminus of the predicted protein and 1H208 containing the carboxy terminus (Fig. 1c). Fusion of the non-overlapping coding sequences of 1H280 and 1H208 yields a predicted full-length protein of 60K.

Amino-acid sequence

The predicted amino-acid sequence of 1H280/1H208 (Fig. 1d) contains a domain clearly related to the winged-helix domain of the forkhead/HNF3 β family of transcription factors. This domain is as similar to members of the forkhead/HNF3 β as previously known members of this family are to one another (42% identity, 58% similarity to rat HNF3 β in the winged-helix domain; Fig. 1d)²⁴, and contains a highly conserved sequence identified in HNF3 β as a nuclear localization signal²⁵. No strong similarities of 1H280/1H208 to any other winged helix protein outside the winged helix domain were found. The predicted N-terminal 12 amino acids are, however, similar to the N terminus of the mouse mesenchyme forkhead (Mfh-1; ref. 26) (50% identity, 58% similarity over the N-terminal 12 amino acids). Because of the potential role of this forkhead/winged-helix factor in the formation of ARF and transduction of activin/Vg-1/TGF- β signals, we have named it forkhead activin signal transducer-1 (FAST-1).

To confirm that the FAST-1 clone identified in the yeast one-hybrid assay binds ARE with specificity similar to that of ARF,

messenger RNA encoding Myc-epitope-tagged FAST-1 was injected into 2-cell frog embryos. EMSA using radiolabelled ARE as probe demonstrated that injection of FAST-1 mRNA resulted in expression of an activity that binds to wild-type ARE but not mutant ARE (Fig. 2a). Overexpressed FAST-1 protein clearly migrated much faster than endogenous ARF, although overexpression of FAST-1 did reproducibly enhance ARF formation in response to activin stimulation. Both the expressed FAST-1 and ARF also failed to bind ARE probe in which only the 5' 6-bp repeat was mutated, confirming the identical site specificity of these two binding activities (not shown). Identical binding results were obtained with overexpressed FAST-1 when the Myc-epitope tag was replaced with the correct N terminus of the protein (from the clone 1H280) (data not shown). Full-length, untagged FAST-1 migrates slightly higher in EMSA than the tagged construct, however, making it impossible to resolve from a major nonspecific DNA-binding activity²⁰ and therefore more difficult to study than the tagged construct. *In vitro* translation of FAST-1 also generates an ARE-specific DNA-binding activity, indicating that post-translational modification of FAST-1 in the embryo is unnecessary for activity (not shown). Clone 1H280, which encodes a protein containing the winged-helix domain but lacking the C-terminal 264 amino acids, binds with the same specificity, suggesting that the winged-helix domain is the major determinant of binding activity and specificity. Overexpressed FAST-1 appears to be constitutively localized to the nucleus of early embryonic animal pole cells (Fig. 2b); this localization is not significantly affected by

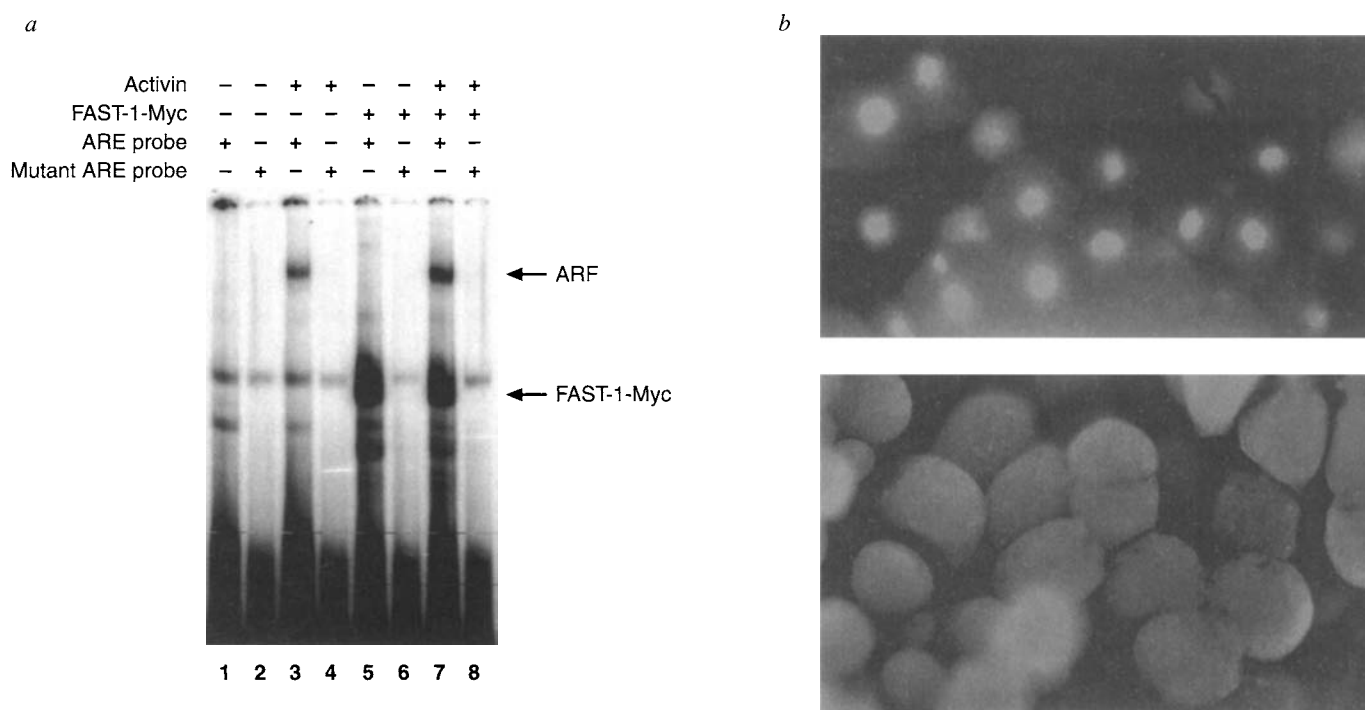


FIG. 2 Activity, localization and developmental expression of FAST-1. *a*, Activity of expressed FAST-1 in EMSA. Uninjected embryos, or embryos injected with mRNAs encoding Myc-tagged FAST-1 or *Xenopus* activin β B were lysed and assayed by EMSA using radiolabelled wild-type or mutant ARE oligonucleotides as probes. *b*, Localization of tagged, expressed FAST-1 to the nucleus. Overexpressed Myc-tagged FAST-1 (top panel) was visualized by anti-Myc immunofluorescence. As a control, fluorescence visualization of cytoplasmically localized p70 S6 kinase was achieved using overexpressed, Myc-tagged p70 S6 kinase (bottom panel). *c*, Developmental expression of FAST-1 mRNA. Northern blot of FAST-1 mRNA expression over a range of developmental stages⁵². The blot reprobed for ornithine decarboxylase (ODC) mRNA is shown as a mRNA-loading control.

stimulation by activin (not shown).

FAST-1 expression in development

ARF can be formed from maternally encoded components in the early embryo²⁰. We examined the developmental expression of FAST-1 mRNA by northern analysis: FAST-1 mRNA is present in oocytes and in early embryos until shortly after gastrulation, after which transcript levels decline and remain low through tadpole development (Fig. 2c). That this distribution reflected FAST-1 mRNA and not crossreaction with homologous forkhead/winged-helix domain transcripts was confirmed by polymerase chain reaction with reverse transcription (RT-PCR) directed against the 3' untranslated region of the FAST-1 transcript (not shown). This expression pattern is consistent with the maternal origin of the constituents of ARF, and coincides well with the timing of expression of *Mix* genes during development, which can be induced as soon as zygotic transcription begins at the mid-blastula transition, declines to low levels at the end of gastrulation and remains low throughout subsequent development^{19,22}.

FAST-1 as component of ARF

To test directly whether FAST-1 was a component of the EMSA factor we had previously identified as ARF, we tested tagged FAST-1 for incorporation into ARF. Myc-epitope²⁷-tagged FAST-1 was expressed in embryos by mRNA microinjection. Overexpression of tagged FAST-1 enhanced the formation of ARF in response to activin stimulation (Fig. 3a) and incubation of the gel-shift mixture with anti-Myc antibody resulted in a shift in the mobility of the ARF complex. Overexpression of untagged FAST-1 also enhanced formation of ARF in response to activin, but this complex did not supershift in the presence of anti-Myc

IgG (not shown). An additional low-mobility band supershifted by anti-Myc IgG but not requiring activin stimulation is probably due to the crosslinking of tagged FAST-1 protein by the bivalent anti-Myc IgG (indicated by arrow labelled ss(FAST-1-Myc)_n in Fig. 3a).

Finally, to confirm that FAST-1 is a major DNA-binding component of ARF, we compared the mobility in denaturing SDS-polyacrylamide gel electrophoresis of FAST-1 crosslinked to ARE with that of the major DNA-binding component of ARF crosslinked to ARE. We bound ARF or FAST-1 to ARE, resolved the resulting complexes by EMSA, irradiated the gel with ultraviolet light, and identified and excised the complexes by autoradiography (Fig. 3b, left). We then resolved proteins crosslinked to the probe within the complex and identified them by SDS-PAGE and autoradiography (Fig. 3b, right panel). In EMSA, full-length FAST-1 comigrates with a nonspecific DNA-binding protein; to distinguish ARE bound to FAST-1 from that bound to the nonspecific binding protein, parallel analyses were done with mutant ARE, which does not bind FAST-1 but does bind the nonspecific factor (Fig. 3b; compare samples 2 and 3). Comparison was also made with the major nonspecific DNA-binding activity bound to wild-type ARE in the absence of expressed FAST-1 (Fig. 3b, sample 4). The protein corresponding to overexpressed FAST-1 crosslinked to the wild-type ARE probe was found to co-migrate with a protein from ARF crosslinked specifically to ARE (Fig. 3b, right panel, compare samples 2 and 1). A second, nonspecific DNA-binding protein crosslinked to ARE electrophoresed at a higher apparent M_r (Fig. 3b, right panel, samples 2, 3, 4). The similar size of the ARE-FAST-1 crosslinked complex and ARE crosslinked to the major DNA-binding component of ARF, in conjunction with the site specificity and

supershift data already described, show that FAST-1 is a major DNA-binding component of ARF.

MAD2 as a component of ARF

Although the properties of FAST-1 identify it as a component of ARF, the low mobility of ARF relative to FAST-1 in EMSA strongly indicated that there are additional components of ARF. Protein products of *Mad* genes have been identified as potential transducers of TGF- β superfamily signals that accumulate in the nucleus after stimulation with the appropriate ligand²⁸⁻³⁰. Nuclear targets for MADs have not been identified; to determine whether a member of the MAD family could interact with FAST-1, the two characterized *Xenopus* MADs, XMAD1 and XMAD2 (ref. 23) were tested as components of ARF. Overexpression of XMAD1 or XMAD2 alone was not sufficient to activate ARF, but ARF formed by activin stimulation in the presence of tagged XMAD2, but not XMAD1, generates a supershift of a major portion of ARF when incubated with anti-Myc antibody (Fig. 4). Equal expression of XMAD1 and XMAD2 was confirmed by anti-Myc western blot (not shown). Overexpression of untagged XMAD1 or XMAD2

and incubation of ARF with anti-Myc antibody did not supershift ARF (Fig. 4). Supershift of ARF without overexpression of XMAD2 was also observed using an antibody³¹ that recognizes a domain common to MADR1 and MADR2 (not shown). Overexpression of an activating truncation of mouse MADR2 (provided by J. Wrana and P. Hoodless) stimulates transcription of *Mix.2*, confirming that activation of this MAD is sufficient for *Mix.2* promoter regulation (not shown). We have previously shown that stimulation of early embryos with Vg-1 by microinjection of the chimaeric construct B-Vg can stimulate ARF formation, as does activin²⁰. This Vg-1-stimulated ARF also incorporates epitope-tagged XMAD2 (not shown). These observations demonstrate that XMAD2, but not XMAD1, can directly participate in the ARF complex in response to activin or Vg-1 stimulation.

Discussion

TGF- β signal transduction. Several components of the pathway by which signals from TGF- β superfamily of factors are transduced have been identified³², but how these components regulate

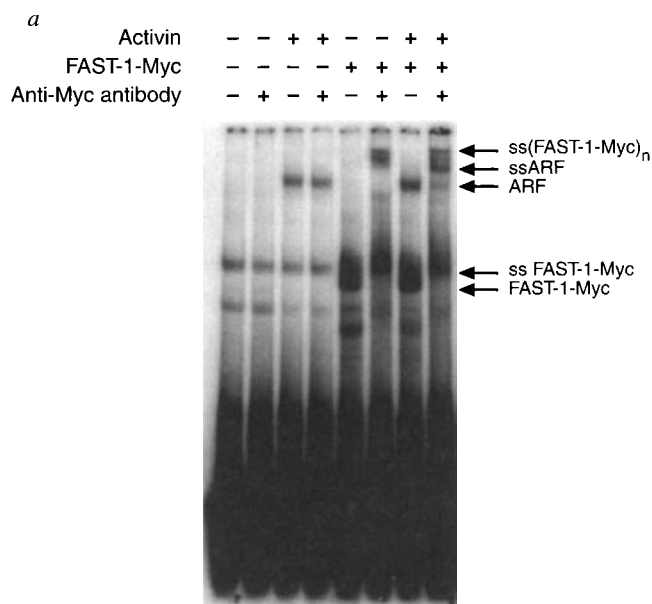
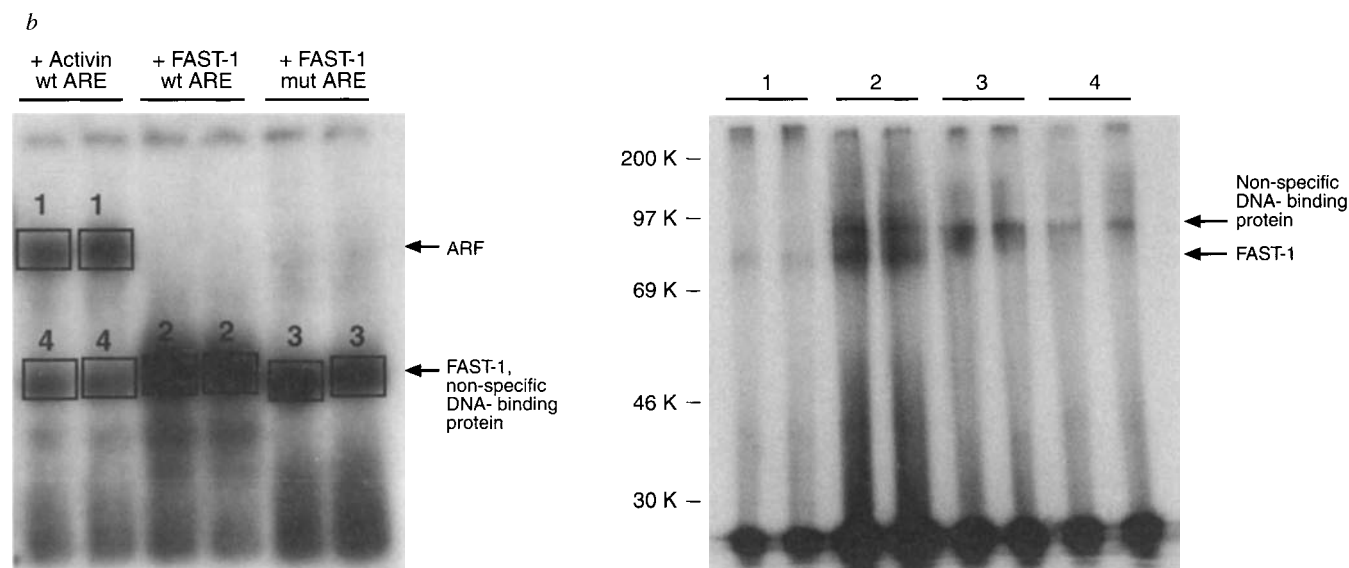


FIG. 3 FAST-1 is a component of ARF. **a**, Supershift of ARF from embryos expressing tagged FAST-1. Embryos injected with mRNA encoding Myc-tagged FAST-1 or activin, or both, were lysed and analysed by EMSA in the absence or presence of anti-Myc antibody. Arrows indicate migration of tagged FAST-1, antibody-bound FAST-1 (ssFAST-1 Myc), ARF, antibody-bound ARF (ssARF) and antibody-crosslinked FAST-1 (ss(FAST-1-Myc)_n). **b**, Crosslinking of ARE to FAST-1 or ARF. In the left panel, lysates are shown from embryos injected either with activin mRNA to activate ARF formation or with FAST-1 mRNA that were assayed by EMSA with wild-type or mutant ARE and irradiated with UV; after autoradiography, the indicated boxes were excised (from duplicate samples) and loaded onto an SDS gel (right) in the lanes indicated. FAST-1 and a major nonspecific DNA-binding activity comigrate in EMSA and are therefore distinguished by the elimination of FAST-1 binding activity when mutant rather than wild-type ARE is used (compare samples 2 and 3).



early transcriptional responses is not yet clear³³⁻³⁹. Genetic screens originally identified *mad*⁴⁰⁻⁴² as a potential signal transducer of *dpp*, a *Drosophila* BMP4 homologue. The products of vertebrate homologues of *mad* are important transducers of TGF- β superfamily signals¹²⁻¹⁵. Overexpression of MAD homologues can mimic the effects of BMP in cultured cells or *Xenopus* embryos^{28-30,43,44}. How could MADs transduce TGF- β signals? They undergo ligand-dependent phosphorylation and nuclear accumulation, and at least one MAD has a latent transcriptional-activation domain²⁸⁻³⁰. It is unknown, however, how the latent MAD transcriptional activation domain is directed to the appropriate promoter element of a responsive gene.

Several vertebrate *MAD* genes, *XMAD2* in *Xenopus* and *madr2* in mouse, may be coupled to activin and TGF- β signalling^{23,29,45}. *Madr2* accumulates in the nucleus in response to activin²⁹, and overexpression of *XMAD2* or *Madr2* induces transcriptional responses similar to those stimulated by activin²³, suggesting that *XMAD2* is a transducer of an activin-like signal in frog embryos. Our results directly support this idea: *XMAD2*, but not the BMP-coupled *XMAD1*, can complex with FAST-1 to form a site-specific transcriptional regulatory complex in response to stimulation by activin (or Vg-1). This confirms that *XMAD2* is in an activin-responsive pathway and suggests a mechanism by which the putative transcriptional regulatory activity of *MAD2* might be directed to the promoter of an activin-responsive gene.

Winged-helix factors and the regulation of transcription. Winged-helix transcription factors are defined by a conserved DNA-binding motif found in the *Drosophila* homeotic gene forkhead and rat hepatocyte nuclear factor-3 (HNF-3 β)^{24,46}; more than 80 genes encoding this conserved domain have been identified²⁴. Many have developmentally specific patterns of transcription and function in the regulation of morphogenesis and cell specification²⁴. FAST-1 does not fall into any of the previously identified subgroups of the winged-helix family²⁴, even though the similarity of the winged-helix motif (58% similarity, 42% identity to the HNF-3 β winged-helix domain) establishes it as a member of this family. A consensus DNA-binding site common to several of the winged-helix factor subgroups has been identified; this consensus site (G/A)(T/C)(C/A)AA(C/T)A is not similar to the 6-bp repeat necessary for FAST-1 factor binding to the ARE, nor is it anywhere in the ARE. This is not unprecedented, however, as several winged-helix factors also bind to consensus sequences entirely different from the HNF-3 β consensus site²⁴. The relatively distant primary sequence relationships between members of the winged-helix family appear to allow too broad a range of possible target sequences to define a consensus target sequence for the entire family.

ARF can be formed in the presence of cycloheximide, and as early as the 8–16-cell stage²⁰. These observations, and the maternal expression of FAST-1 mRNA, indicate that FAST-1 is constitutively present throughout early cleavage stages. FAST-1 DNA-binding activity, however, is not detectable in early embryos. Although two factors that bind to ARE are detectable in lysates from early embryos (Fig. 1), one of these is a nonspecific DNA-binding protein and the other binds with a site specificity distinct from that of FAST-1 or ARF (X.C. and M.W., unpublished). The nonspecific DNA-binding activity comigrates with full-length FAST-1, raising the possibility that FAST-1 is masked by nonspecific DNA binding in our EMSA. Alternatively, FAST-1 DNA-

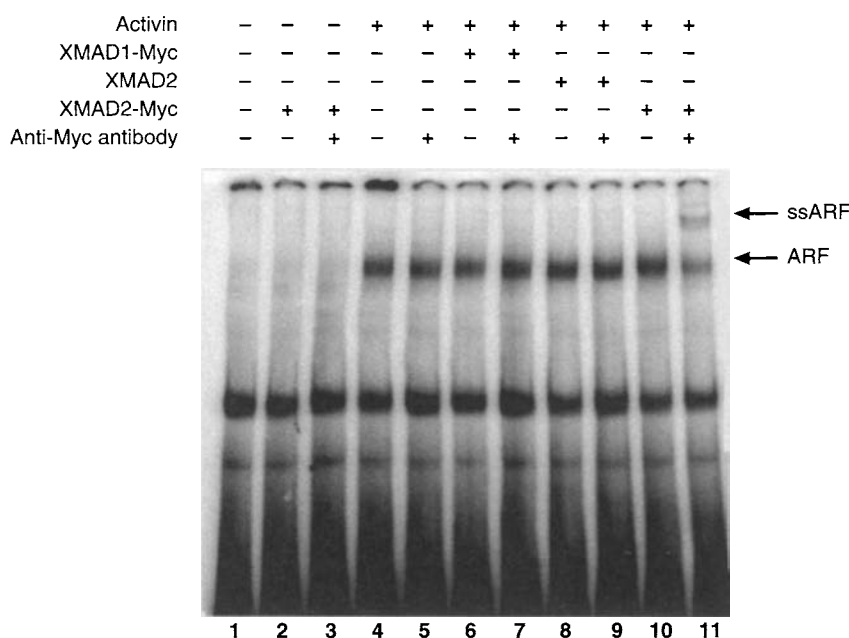


FIG. 4 *MAD2* is a component of ARF. Supershift of ARF from embryos expressing epitope-tagged *MAD2*. Embryos expressing tagged or untagged *XMAD1* or *XMAD2* in the presence or absence of activin were assayed by EMSA in the presence or absence of anti-Myc antibody as for Fig. 3. Arrows indicate ARF or antibody-bound ARF (ssARF).

binding activity may be negatively regulated *in vivo*; in this case *XMAD2* or an additional activin/Vg-1-dependent signal may relieve this inhibition to stimulate DNA binding. Overexpressed FAST-1 is constitutively active as an ARE-specific DNA-binding protein in embryo extracts, but overexpression may saturate endogenous inhibitors of FAST-1. Overexpressed FAST-1 does not activate *Mix.2* transcription (X.C. and M.W., unpublished observations), indicating that binding of overexpressed FAST-1 to ARE in the endogenous *Mix.2* promoter is not sufficient for activation of transcription. *XMAD2* binding to FAST-1 may be necessary for transcriptional activation by the ARF complex; the observation that *MAD1* can activate transcription when fused to a DNA-binding domain³⁰ is consistent with this possibility. The N-terminal portion of FAST-1 (clone 1H-280), however, is sufficient to activate transcription of a yeast *lacZ* reporter, indicating that FAST-1 itself contains a latent transcriptional-activation domain, and may therefore participate in transcriptional activation as well as DNA binding.

Activin/Mad/FAST-1 regulation of *Mix.2* transcription. *XMAD2* and FAST-1 associate in an activin-stimulated DNA-binding complex to mediate the transduction of a TGF- β superfamily signal. A model summarizing the simplest interpretation of the available data is shown in Fig. 5, in which activated *MAD2* translocates to the nucleus and associates with FAST-1 to generate a transcriptional activation complex. Several important questions remain: *XMAD2* and FAST-1 appear to be components of the activin-responsive factor we previously identified, but are they the only components of this complex? How many of each molecule are present in the complex? How is the recruitment of *XMAD2*, FAST-1 and any additional components into the ARF complex regulated? A possible answer for this last question, suggested by the work of Hoodless *et al.*²⁸, is that ligand-stimulated phosphorylation of *XMAD2* drives its association with FAST-1 or other components of ARF. But this does not exclude the possibility that FAST-1 or additional components are regulated by an *XMAD2*-independent pathway as well.

We have presented evidence that FAST-1 and *XMAD2* are involved in the regulation of *Mix.2* transcription by activin-like

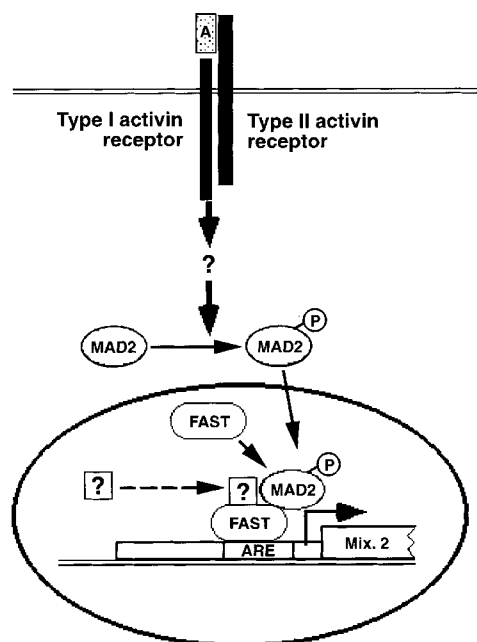


FIG. 5 Model for activin ('A' in figure) activation of *Mix.2* transcription in the early embryo.

signals. Other evidence indicates that members of the MAD family are transducers of a number of transcriptional responses in response to several TGF- β superfamily members. The highly specific expression patterns of winged-helix factors are consistent with these factors mediating tissue-specific responses to TGF- β superfamily members by association with MAD proteins. On the other hand, the winged-helix domain itself is thought to be primarily involved in DNA binding rather than in protein-protein

interaction, so the non-conserved sequences outside this domain may determine the association of FAST-1 with XMAD2. The only other activin-responsive element from frog embryos shares no sequence similarity with our ARE⁴⁷, indicating that a variety of target sites and corresponding transcription factors are involved in mediating early embryonic activin responses. Other examples of promoter-specific TGF- β regulation will establish whether or not the winged-helix domain is a common feature of TGF- β -regulated transcription factors. Our results indicate that a direct pathway linking activation of a cytosolic signal transducer to a site-specific DNA-binding protein may mediate transcriptional responses to TGF- β superfamily members. □

Methods

Electrophoretic mobility-shift assay. EMSA was done on lysates from stage 8–9 uninjected or activin-injected embryos as described²⁰. For supershift assays, EMSAs were carried out as usual except assay mixtures were incubated with anti-Myc antibody 9E10 for 1 h on ice before gel loading. For ultraviolet crosslinking, BrdU-labelled wild-type or mutant ARE probe was used; gels were irradiated for 40 min on a 310 nm transilluminator and autoradiographed without fixing or drying. Bands were excised, soaked in SDS-sample buffer and loaded directly onto a 10% SDS-polyacrylamide gel.

One-hybrid screen. A yeast one-hybrid assay^{48,49} was used to identify proteins capable of interacting with trimerized ARE. Yeast were transformed and screened with a *Xenopus* oocyte cDNA pGAD10 fusion library (Clontech; used according to the manufacturer's instructions). 48 clones were isolated by virtue of activation of the expression of both reporter genes, *HIS3* and *lacZ*, in the host yeast strain. To confirm the binding specificity for ARE, plasmid DNA was isolated from putative positive clones and individually re-transformed into host strains containing either wild-type or mutant trimerized ARE upstream of a *lacZ* reporter. ARE-specific binding activities were then identified on X-Gal plates as colonies that were blue in the host carrying the wild-type reporter construct and white in the host carrying the mutant reporter construct.

Immunofluorescence. Whole-mount immunofluorescence was done on animal caps dissected from Myc-FAST or Myc p70, S6 kinase RNA-injected stage 8.5 embryos fixed in MEMFA and probed essentially as described in ref. 50, using the anti-Myc monoclonal antibody 9E10 and rhodamine donkey anti-mouse second antibody.

Northern blots. Northern blots were done on whole RNA extracted from frog embryos as described⁵¹ using antisense RNA probes.

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Total ablation of the debris from the 1908 Tunguska explosion

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THE origin of the explosion over Tunguska, central Siberia, in 1908 has long been an enigma. Models^{1–3} of the disruption of solid objects entering the atmosphere indicate that the Tunguska explosion occurred at an altitude of 6–10 km, and that the source object was probably a stony asteroid⁴. But important questions concerning the nature of the object remain^{5,6}, particularly as no fragments have been identified in the area of the explosion. Unlike smaller objects (such as meteorites), which decelerate high in the atmosphere and can thus escape complete ablation and/or pulverization⁷, a Tunguska-sized object penetrates deeper into the atmosphere, where it will experience a greater aerodynamic load: the object should be disrupted into a vast number of fragments, each no larger than about 10 cm (ref. 2), which are then widely dispersed. Here I calculate the flux of radiation both inside and outside the fireball associated with the fragmenting object, and show that this is sufficient to totally ablate the dispersing fragments. The apparent absence of solid debris is therefore to be expected following the atmospheric fragmentation of a large stony asteroid.

On 30 June 1908, an unknown cosmic body came down to Earth in the basin of the Podkamennaja Tunguska, central Siberia. The main characteristics of the explosion—coordinates of epicentre, height, and energy yield (10–20 megatons)—have been deduced from seismic and atmospheric wave records and data on forest devastation. The absence of craters and meteorite fragments was easily explained by a cometary hypothesis, but theoretical investigations show that a representative comet of appropriate kinetic energy would explode too high in the atmosphere^{1,2}. Cometary origin, however, cannot be fully rejected because the trajectory inclination and entry velocity of the Tunguska object, as well as the density and composition of comets, are uncertain. Proponents of asteroidal origin of the Tunguska bolide inevitably imply that this impactor broke up and was pulverized in the atmosphere⁸. To investigate the problem quantitatively, it is necessary to estimate the sizes of the bolide fragments and radiation flux on them.

Korobeinikov *et al.*⁹ computed the radiation impulse on the ground on the assumption that the Tunguska event was a combination of spherical and cylindrical (with constant specific energy) explosions. The model of Chyba *et al.*⁴ gives energy release along the trajectory which can be treated as a line source of variable

specific energy $E(l)$, where l is the coordinate measured along the trajectory. The radiation flux at some point $\mathbf{r}$ can be approximated as

$$q(\mathbf{r}, t) = \int_0^{L(t)} f(l, t) E(l) \frac{\cos \alpha}{4\pi D^2} e^{-k \int_0^D ds} dl \quad (1)$$

where t is time, L the length of the trajectory, f the portion of energy radiated per unit time, D the distance from the trajectory point to $\mathbf{r}$, α the angle between the normal in relation to the illuminated surface and the ray directed to the trajectory point, k the averaged absorption coefficient of the undisturbed atmosphere¹⁰, d the ratio of atmospheric density to that at the sea level and s the coordinate along the ray. It was assumed that this function is identical to that of a cylinder-shaped explosion initiated by a cylinder enlarging its radius—the velocity and the maximum radius of the cylinder being equal to those of the meteoroid. Radiation transfer was computed with a multi-group approximation¹¹.

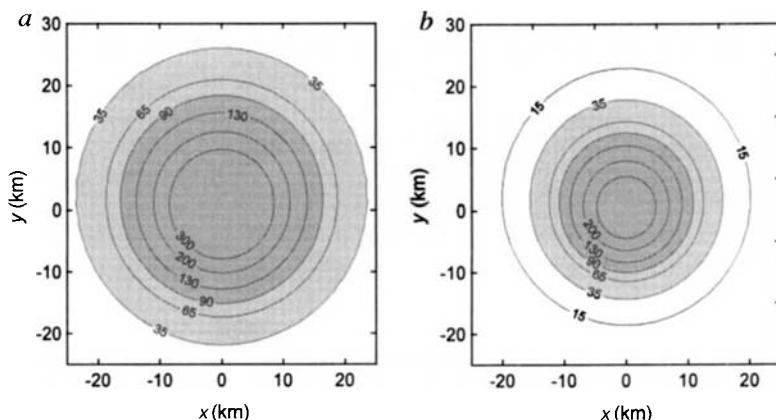
The calculations were made for 15-megaton asteroid-like object⁴ with an initial velocity of 15 km s^{-1} and an incidence angle of 45° . Maximum radiant exposure at the ground is shown in Fig. 1. The expeditions in the 1920s and 1930s reported that the average radius of the area of charred trees and soil was $\sim 15 \text{ km}$ with a scorch spread of up to $\sim 20 \text{ km}$, gradually decreasing at large distances^{12–14}. Computations for fine weather are consistent with these findings—about $40\text{--}60 \text{ J cm}^{-2}$ are required for visible charring of wood¹⁰.

Intense light flashes have been registered by satellite-based optical sensors^{15,16}. In this connection, a light curve of the Tunguska event seen from an infinite distance has been computed (Fig. 2). Growth rate of the main impulse is in line with the most powerful of the registered flashes, which additionally supports the idea of a pancake spreading impactor⁴.

Nevertheless, the behaviour of a heavily fragmented meteoroid is not so simple as the semi-analytic models^{1–4} predict. Rayleigh–Taylor instabilities (growing at the rate the idealised pancake impactor grows) lead to disintegration and spreading of the fluid impactor. Hydrocodes show that the meteoroid takes an irregular shape^{17,18} and fully pulverizes at some point in time¹⁸ and repulsive forces acting between the fragments^{19,20} produce a swarm of debris. Some results of hydrodynamic modelling¹⁸ for the late stage of disintegration are demonstrated in Fig. 3.

Fragmentation starts at a height of 25 km when the ram pressure exceeds the yield strength⁴. The pressure reaches its peak, $8 \times 10^8 \text{ dyn cm}^{-2}$, at about 10 km. Let us assume: (1) that the strength of the object diminishes with increasing size (scaling effect)^{21,22}; (2) the strength of centimetre stony samples is $1\text{--}6 \times 10^8 \text{ dyn cm}^{-2}$ (ref. 23); and (3) that smaller fragments lag behind²² while large ones take on the brunt of the aerodynamic load. Then, equating the fragment strength to the maximum

FIG. 1 Isolines of radiation energy absorbed by a unit area at the ground in J cm^{-2} . It is assumed that the irradiated surface is best orientated to accept the maximum radiation, that is, the isolines indicate a zone with the greatest possible heat damage. Results of computations are shown for atmospheric visibility up to a, 40 km (very clear, $k = 0.1 \text{ km}^{-1}$ (ref. 10)) and b, for the visibility of 20 km (clear, $k = 0.2 \text{ km}^{-1}$). The shaded area lies within the threshold for setting fires. The y-axis is a projection of the bolide trajectory to the Earth's surface. The coordinates start at the epicentre—the z-axis meets the trajectory at an altitude of 7 km. The computational results give the best fit to the evidence^{12–14} at weather conditions between very clear and clear. The maximum radiant exposure at 70 km to the south-east is 0.55 J cm^{-2} for 40 km visibility and 0.033 J cm^{-2} for 20 km visibility. That is also in reasonable agreement with eyewitness reports from Vanovara²⁹.



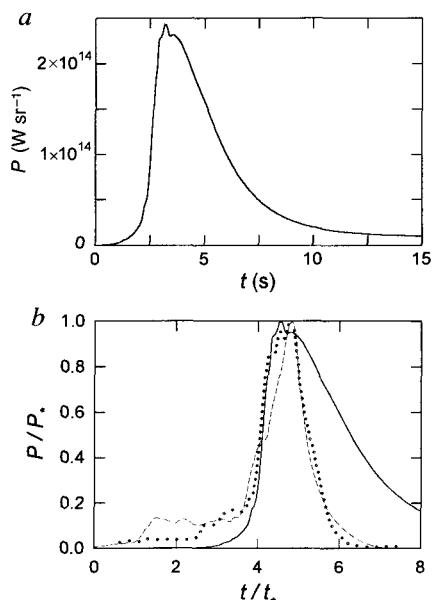


FIG. 2 *a*, Radiation power per unit solid angle versus time as it would be seen from a satellite viewing the Tunguska explosion. *b*, The Tunguska radiation impulse is compared with events registered by satellite based sensors on 1 October 1990 (dotted line) and 1 February 1994 (dashed line). The following nondimensional units are used in the latter case: P_* is the maximum of radiation power, t_* is the scale time equal to E/P_* , where E is the energy radiated from the beginning of the impulse till its peak. Growth rates of the main flashes differ insignificantly. A rise in light power before the main flash in the registered light curves shows that the breakup can go through several stages. The Tunguska light curve decline rate is lower because of the much higher energy and a lesser altitude of the Tunguska explosion. Initial kinetic energies of the impactors were about 6 kilotons and 40 kilotons in the 1 October 1990 and the 1 February 1994 events respectively, assuming a 10% conversion of kinetic energy to radiation energy¹⁶. The source height at peak power is about 30 km for the 1 October 1990 and about 21 km for the 1 February 1994 events. A calculated coefficient of conversion for kinetic energy to radiation for the hypothetical Tunguska event is 20%, twice that for the events with smaller energy due to much longer decline part of the light curve. The whole impulse lasts for about 20 s in agreement with observational data for powerful nuclear explosions in the atmosphere¹⁰. The radiation efficiency of the Tunguska bolide is much higher than empirical radiation efficiencies for small meteors³⁰ ($\sim 1\%$) due to greater optical thickness of the Tunguska fireball, but it is lower than thermal partition of a nuclear explosion^{10,31} (35–43%) owing to lower temperatures. Dust and vapour, especially metallic components, may change the opacities and radiation efficiencies obtained here.

FIG. 3 A swarm of debris at full disintegration stage of the Tunguska meteoroid. Numerical results were initially obtained for a 2-km fragment of the comet Shoemaker–Levy 9 (ref. 18) and then converted to the 58-m Tunguska object on the assumption of hydrodynamic similarity and the pancake model applicability. Dramatic deceleration occurs within a much shorter distance than the scale height of the atmosphere for both of the events. Solid circles are particles of the meteoroid, points are particles of the atmosphere. The regular pattern of points corresponds to the undisturbed atmosphere. The hypothetical meteoroid initially consisted of 650 particles, but only 340 particles are confined to computational region shown in the figure. The rest are decelerated in the far wake. Particles located between leading fragments and the cross-section at a 300 m distance from it have, in average, 65% of the initial velocity, that is $\sim 10 \text{ km s}^{-1}$. More massive fragments hold a leading position. The meteoroid breakdown into small segregated debris suggests a large increase in the total surface. Ablation rate at the disintegration stage becomes much greater than the single-body models^{2,4,5} predict.

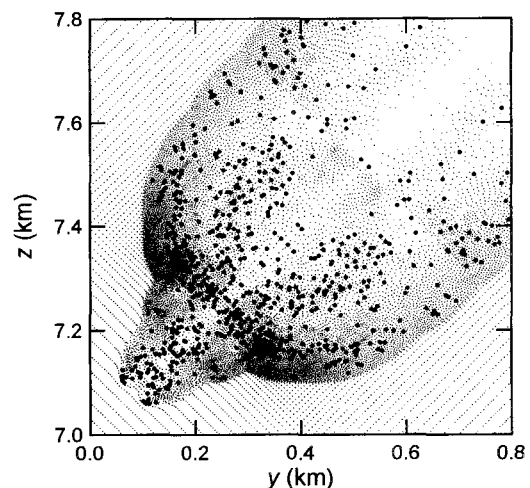
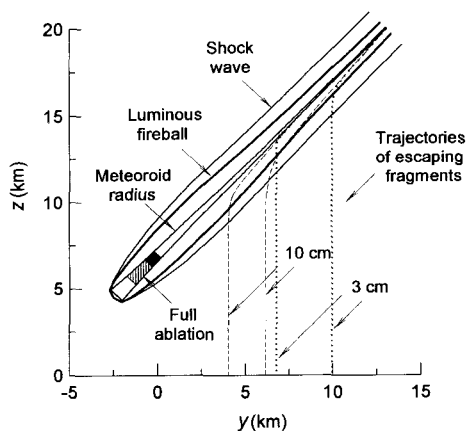


FIG. 4 The shape of the shock wave and the fireball; the bolide's head velocity is down to 3 km s^{-1} . The computational results are obtained using the spreading impactor model⁴ and treating the energy release as a linear source. The increasing radius of meteoroid is plotted inside the fireball. The luminous region boundary is defined as an isotherm of 2,500 K temperature. Computations of fragment motion and ablation show that 3-cm fragments fully ablate as they begin to move separately inside the area shown by hatching. Fragments 10 cm in size ablate if they are segregated inside or above the shaded area. Above the shaded zone, the bolide is heavily fragmented but the fragments are not completely dispersed. It is highly probable that the dispersion occurs at the upper boundary of the shaded region. Trajectories of fragments that escape the fireball at altitudes of 15 to 20 km at a lateral velocity of 1 km s^{-1} are shown by dashed lines for 10-cm fragments and by dotted lines for 3-cm fragments. It is possible that remnants of such fragments could reach the ground at a distance of 5 to 10 km from the epicentre. Fragments 3 cm in size escaping the fireball at altitudes below 14 km do not survive the fall because of the sufficiently high radiation flux (about 10^5 W cm^{-2}) on their surface. Larger (10 cm) fragments escaping from the fireball at altitudes below 15 km could withstand radiation from the fireball. It is unlikely, however, that they survive because they are weaker (because of the scaling effect) and would be broken up at aerodynamic loads greater than $4 \times 10^8 \text{ dyn cm}^{-2}$.



pressure, we find that sizes of surviving fragments are less than 1–3 cm. A fragmentation model of Hills and Goda² at constant strength predicts the largest fragment mass to be ~1 kg, which implies that the fragment size is under 10 cm for the stony impactor considered here.

Change in mass, m , of a single fragment is given by

$$Q \frac{dm}{dt} = -qA \quad (2)$$

where Q is the heat of ablation, q the radiation flux density on the surface, and A the surface area—full surface if the fragment is entirely immersed in the fireball, or cross-section if it is irradiated from one side. Despite the fact that $Q = 8 \text{ kJ g}^{-1}$ on vaporization, a value of $Q = 2 \text{ kJ g}^{-1}$ is more suitable for meteoroids owing to instabilities in melted layers, inhomogeneities, and existence of volatiles in stony meteoroids²⁴ (the heat transfer coefficient, commonly involved in equation (2), is not used here because q is evaluated directly.) For a fragment inside the swarm, q is about the radiation flux of black-body with a temperature equal to that behind the shock wave of the swarm if the velocity is from 10 km s^{-1} to 4 km s^{-1} . Free paths of photons are from 0.1 cm to 100 m in this case²⁵, less than the fireball size but greater than the screening layer around the fragment. Free paths of photons depend on the temperature and density of the air. For velocities below 10 km s^{-1} , the temperatures are less than 15,000 K, the air is partially ionized and the opacities are determined mainly by bound–free and free–free electron transitions. Screening by vapour is negligible because the vapour mainly absorbs hard photons, with energies above 7.5 eV (ref. 26).

Some fragments can escape the fireball as a result of accidental collisions. The radiation flux q is then determined by equation (1) if the fragment velocity falls below 7 km s^{-1} —the effect of screening of soft fireball radiation by the shock-heated cap around the fragment disappears. If we take up these assumptions, the computations show that 3–10 cm fragments fully ablate inside or outside the fireball unless the altitude at which they gain significant lateral velocity is high enough (Fig. 4). Conceivably, micro-sized particles trapped by resin of trees^{27,28} might be recondensed material precipitated in the general vicinity of the impact site. Similar to the impact of the comet Shoemaker–Levy 9, Tunguska debris was probably also widely scattered because of the wake, a rarefied channel made by the bolide in the atmosphere. Two essential properties of a large bolide lead to the result reported here: fragment sizes are much less than they are for small impactors and, second, radiation energy is decidedly large—quite comparable with that of a nuclear explosion. □

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Two-dimensional photonic-bandgap structures operating at near-infrared wavelengths

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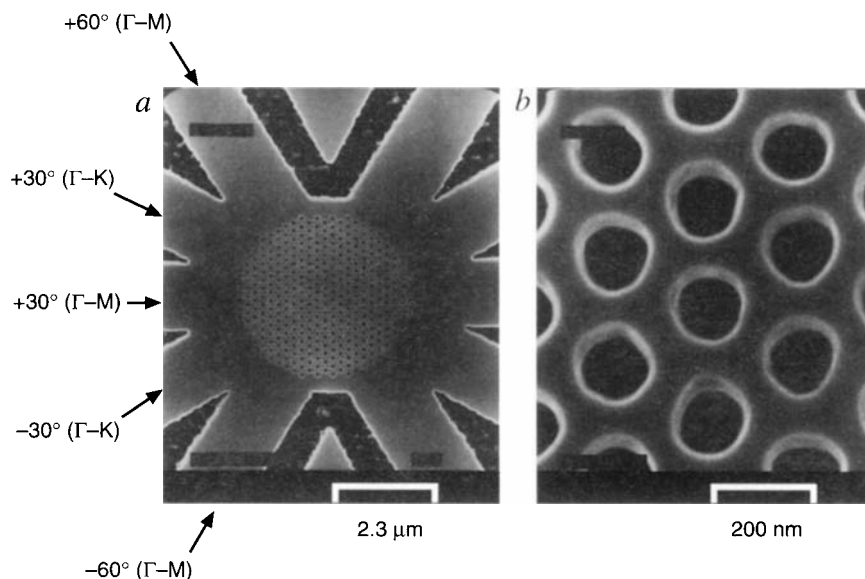
PHOTONIC crystals are artificial structures having a periodic dielectric structure designed to influence the behaviour of photons in much the same way that the crystal structure of a semiconductor affects the properties of electrons¹. In particular, photonic crystals forbid propagation of photons having a certain range of energies (known as a photonic bandgap), a property that could be incorporated in the design of novel optoelectronic devices². Following the demonstration of a material with a full photonic bandgap at microwave frequencies³, there has been considerable progress in the fabrication of three-dimensional photonic crystals with operational wavelengths as short as $1.5 \mu\text{m}$ (ref. 4), although the optical properties of such structures are still far from ideal⁵. Here we show that, by restricting the geometry of the photonic crystal to two dimensions (in a waveguide configuration), structures with polarization-sensitive photonic bandgaps at still lower wavelengths (in the range 800–900 nm) can be readily fabricated. Our approach should permit the straightforward integration of photonic-bandgap structures with other optical and optoelectronic devices.

The main factors that determine the properties of photonic-bandgap (PBG) structures are the refractive-index contrast, the fraction of high- and low-index materials in the lattice and the arrangement of the lattice elements. Band-structure calculations show that lattices arranged like a 'honeycomb'^{6,7} or like the atoms in a 'graphite'^{7,8} structure are the most favoured arrangements and predict that the widest bandgap occurs when small regions of high-index semiconductor are surrounded by large regions of air, that is, when the semiconductor 'area fill-factor' is low.

In two-dimensional structures, an additional factor is light confinement; either (1) the structure must be big compared to the beam size or (2) the beam must be sufficiently confined within a waveguide to allow the multiple coherent back-reflections that are required for a PBG. Considering the difficulty of defining periodic structures with sub-100-nm feature sizes, it is beyond current technological capabilities to translate such a pattern into a dielectric matrix many wavelengths deep, so solution (1) is currently not possible at optical wavelengths. Solution (2) has already been realized with semiconductor waveguides^{9–11} and was adopted here.

The waveguide configuration used is comparable to that of a semiconductor laser⁹. A matrix of very small (~100 nm diameter) holes was etched into the waveguide, through the guiding core, to form a honeycomb lattice (Fig. 1). The main difference between our approach and that of others is that the semiconductor area fill-factor is high, so light is confined by a waveguide over most of the propagation path and the strong PBG effects can take place although the depth of the structure is less than the free-space wavelength. If, instead, we adopted the large air-fill factor lattices suggested by theory, the light would only be guided in a small part

FIG. 1 'Star' configuration to probe the transmission properties of the two-dimensional photonic lattice detailed in *b*. Light from a tunable Ti:sapphire laser (practical tuning range here, 820–900 nm) is fed into one of the waveguides on the left-hand side, interacts with the lattice and is picked up by the corresponding waveguide on the right. The waveguide was grown by molecular beam epitaxy and consists of a 12% AlGaAs core, 0.4 μm thick, with a 35% AlGaAs cladding on one side and air on the other⁹. The absorption edge for the 12% AlGaAs material is ~ 800 nm wavelength, that is, transmission is limited to wavelengths of 800 nm and above. The microstructure was fabricated by electron-beam lithography, transfer of the resist profile into SiO_2 and reactive ion etching of the semiconductor using SiCl_4 (ref. 17). The lattice shown here has a period of 190 nm, the diameter of the etched holes is 105 nm (which corresponds to 27% air in the lattice) and the etch depth is 0.55 μm . The centre waveguide (0° angle) probes the lattice in the Γ – M direction and the waveguide above and below ($+30^\circ$ and -30°) in Γ – K . The waveguides at $\pm 60^\circ$ are added to confirm the rotational symmetry of the lattice.



of the lattice and most propagating modes would suffer excessively large diffraction losses at each solid–air interface (except, possibly, for selected Bloch modes^{10,12}). The disadvantage of our approach is that it does not offer the widest possible bandgap and allows bandgaps for only one polarization. Following a proof-of-principle experiment with a high contrast one-dimensional photonic microstructure, however, this is the only approach we are aware of where sharp band edges have been observed at optical wavelengths⁹.

The objective was to perform transmission measurements and observe a range of wavelengths with low transmission (that is, a stopband) with short- and long-wavelength band edges (regions where the transmission changes from high to low or *vice versa*), similar to the results obtained in the microwave regime¹³. Coherent sources in the optical regime, however, do not have the tuning range of their microwave counterparts. In particular, the tuning range of the Ti:sapphire laser we employed is limited to 820–900 nm, which is less than the width of the expected stopbands, so we fabricated a range of lattices with different periods in order to sample different parts of the stopband. Because the stopband position scales with the lattice period, a shift of the lattice period is equivalent to a wavelength shift. We made periods of $a = 180$ nm, 190 nm, ... 220 nm with an area fill-factor of 25–30% air, which results in a smallest hole diameter of 100 nm for the 180-nm lattice. By placing the lattices into a 'star'-shaped stripe waveguide configuration (Fig. 1*a*), we could probe the transmission along different symmetry directions. Although a given lattice may have stopbands in certain directions, a full bandgap only exists if these stopbands overlap for all directions. In the case of a hexagonal close-packed (h.c.p.) lattice, the two directions that differ most are normally known as Γ – K and Γ – M (Fig. 2*a*) and are separated by 30° , as the lattice repeats itself for 60° rotation. For a full characterization, it is sufficient to measure the transmission along these two directions. Furthermore, the vector nature of light causes the response of the lattices to be different depending on polarization, so the measurement must also be polarization-sensitive.

We measured clear band edges for both polarizations, in TE (H-field parallel to etched columns) and TM (E-field parallel to etched columns) (Fig. 2). We shall address TE first. From band-structure calculations⁶, the overlap of the stopbands in the Γ – M and Γ – K directions (that is, a bandgap) was expected. The position of the band edges was designed to be well within the range of the 180–220 nm lattices, that is, the smaller periods were expected to show the long-wavelength band edge and the bigger ones the short-wavelength band edge. But owing to an 'oxide skin' (which was indicated by one-dimensional measurements that

were performed simultaneously¹⁴) the position of the band edges shifted and the bandgap became wider, so we were only able to observe the long-wavelength band edge. The skin is tentatively attributed to a combination of spontaneous oxidation, the addition of oxygen to the etching plasma and amorphization caused by dry etch damage. We assumed a refractive index of $n = 1.5$, a typical value for semiconductor oxides¹⁵, and a thickness of 14 nm, which led to good agreement of the experimentally observed band edges with band-structure calculations (Figs 2, 3).

The results for TM polarization are more subtle; a simple analysis of the experimental data would conclude that there is a full bandgap around 850 nm, as there is a short-wavelength band edge in the Γ – M and a long-wavelength band edge in the Γ – K direction, with no transmission in any direction between 840 and 860 nm. This conclusion, however, is not consistent with theoretical calculations (Fig. 3) and requires further investigation. There is an apparent disagreement with the long-wavelength band edge in the Γ – K direction, which can only be explained when the symmetry of the field profiles is taken into account. In a simple analysis, one might expect transmission at all wavelengths, as band 1 and band 2 meet at the K-point (Fig. 3*a*), so there is a continuum of states available. At short wavelength, the waveguide mode might be expected to couple to band 2 and at longer wavelength to band 1. Instead, only the long-wavelength band edge at 868 nm is obvious, with no apparent transmission at shorter wavelengths. This type of behaviour—involving the failure to observe transmission through states which are clearly present in the band-structure calculations—was originally discussed by Robertson *et al.*¹⁶ for the case of a simple square lattice and is due to the mismatch between the field pattern of the incoming electromagnetic wave and the field pattern of the allowed mode within the structure.

The nature of our measurement is such that the light in the input waveguide needs to couple with the lattice or 'Bloch' mode^{10,12}, travel through the lattice and couple to the output waveguide mode. Consequently, there are two possibilities for low transmission: (1) there is no available lattice mode (a photonic bandgap exists) or (2) the field distribution of the lattice mode does not allow coupling with the waveguide mode. We can clearly see from Fig. 3 that there is no PBG in the Γ – K direction and hence (2) must be the correct explanation. Figure 4*a* shows that the lattice mode of band 1 has the same symmetry as a waveguide mode (alternating positive and negative lobes in the propagation direction with intermediate nodal lines) and should therefore readily couple. The mode in band 2 (Fig. 4*b*), however, has alternating positive and negative lobes in the x -direction, parallel

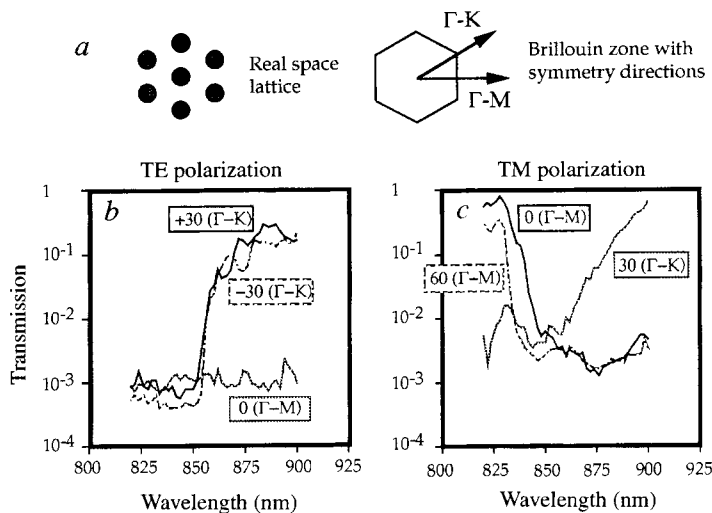


FIG. 2 Sketch of the real space lattice and the Brillouin zone, to illustrate the symmetry directions in the hexagonal close packed (h.c.p.) lattice. The reciprocal space notation is very useful for the description of periodic structures¹⁸. *b*, *c*, Transmission measurement on the 190-nm photonic lattice. *b*, For TE polarization, there is a long-wavelength band edge in Γ -K direction at 860 nm. A similar band edge was observed at 830 nm for a

180-nm lattice and at 895 nm for a 200-nm lattice, which corresponds to the calculation (Fig. 3) within experimental error. The short-wavelength band edge in Γ -K, and both band edges in Γ -M, were not observed for any of the microstructures (between 180 and 220 nm period), which is also in agreement with the bandstructure calculations of Fig. 3. *b*, For TM, the measurement shows a short-wavelength band edge around 840 nm and a long-wavelength band edge around 860 nm. It does not, however, show the short-wavelength transmission in the Γ -K direction that might be expected from the calculations (see text and Fig. 4 for explanation). A similar transmission minimum is found around 890 nm for the 200-nm lattice. The notation Γ -M (0) and Γ -M (60) refers to the 0° and 60° waveguides (Fig. 1) that nominally probe identical directions of the lattice. Considering that the pattern was generated on a cartesian grid and written by an electron-beam writer of limited resolution and accuracy, the small discrepancy between the two directions is easily understood. The excellent overlap between the $\pm 30^\circ$ directions shows that the machine-induced distortions are negligible. The measurement was performed with an unpolarized input beam and a polarizer at the output; the same characteristics, however, were observed for polarized input, that is, we did not observe any polarization conversion in these lattices. The dynamic range of the measurement (note the log scale) and the sharpness of the band edges are also noteworthy and underline the viability of our all-semiconductor waveguide approach; the diffraction loss in the lattice must be very low, otherwise the maximum transmission would not be as high as the 30–50% observed, nor would the wavelength dependence be as sharp. The transmission data is normalized to an identical 'star' configuration without holes etched.

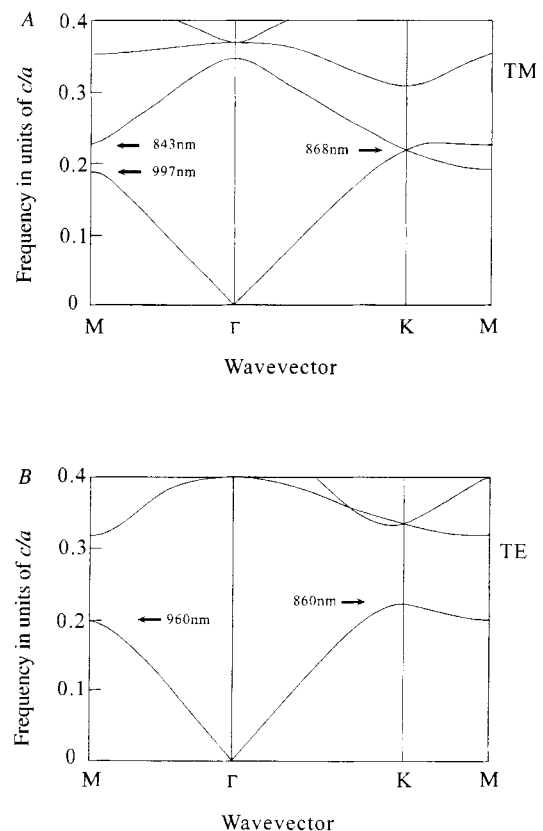
to the propagation direction, indicating that coupling with the incoming plane wave will not occur. Hence, transmission can not be expected although the structure supports a true lattice mode.

Overall, the full, polarization-independent stopband we observe is partially an artefact of the measurement and we can only confirm a sizeable bandgap in TE polarization. Although we did not observe the full stopband in TE owing to experimental limitations, a further field analysis revealed that the states in the

missing short-wavelength band have similar symmetry as the corresponding states in TM, to which coupling was observed.

For active semiconductor device applications, it is possible to engineer polarization selectivity into the structure, so a polarization-specific bandgap can be usefully exploited; PBG structures can be used as mirrors and elements for spontaneous emission control in semiconductor lasers. Furthermore, the demonstration of PBG concepts in a waveguide geometry will allow the realization

FIG. 3 Band-structure calculations for an infinite version of the photonic lattice shown in Fig. 1, assuming 27% air (refractive index $n = 1$), 16% oxide ($n = 1.5$) and 57% semiconductor ($n = 3.42$). The curves show the allowed electromagnetic wave frequencies (which correspond to energies according to the fundamental relationship $E = h\nu$ (ref. 19) as a function of wavevector in the propagation directions shown in Fig. 1a. Regions in which there are no allowed frequencies indicate the existence of a bandgap or stopband which can give rise to a full photonic bandgap (PBG) if this extends over all directions in wavevector space. The bands are numbered from the bottom, so the lowest band is number 1. The band edges, the points where the behaviour changes from propagation to non-propagation, are of particular interest and manifest themselves in the measurement as changes from high to low transmission (or vice versa). They have been indicated for the two main symmetry directions and the arrows refer to the expected band-edge wavelengths for the 190-nm lattice of Fig. 1. The calculations show the expected degeneracy at the K-point for TM polarization (A) and a large bandgap ($\Delta f/f \approx 0.3$) in the case of TE polarization (B). The calculations were similar to those of Pilihal and Maradudin²⁰ and employed 465 plane waves.



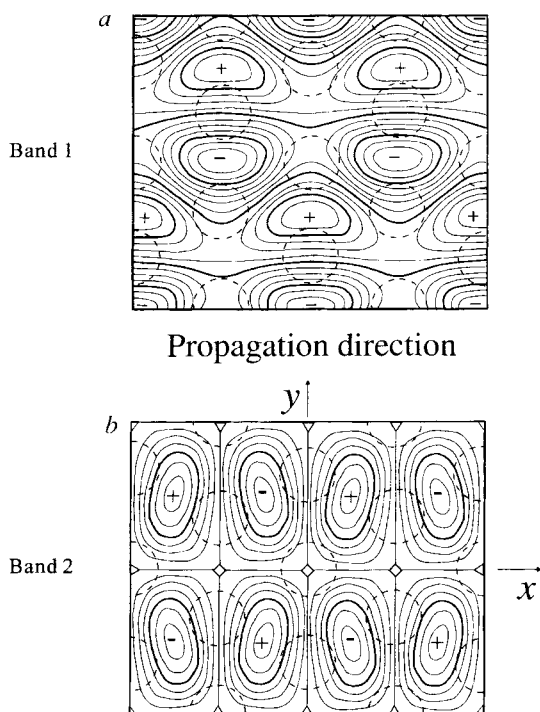


FIG. 4 Snapshots in time of electric-field profiles of the travelling waves corresponding to band 1 (a) and band 2 (b) near the K-point for TM polarization. The (real space) lattice is indicated by dashed circles, showing one complete h.c.p. unit cell as in Fig. 1b. The profiles indicate the different nature of the two bands. In a, although the field profile does not immediately resemble a plane wave, there are continuous regions of positive and negative electric field normal to the propagation direction, which are separated by nodal lines. The field has the same reflection symmetry with respect to the y - z plane as is characteristic of a plane wave, so should readily couple with an incoming plane wave. In contrast, the field in b exhibits a sign reversal asymmetry which does not allow coupling with the incoming plane wave.

of very compact integrated optical elements, such as couplers, switches and wavelength-selective routers. By including localized phase shifts (defects) into the lattice, a novel type of microcavity can be produced¹⁴ and light can be highly concentrated in order to exploit nonlinear effects. □

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X-ray diffraction and equation of state of hydrogen at megabar pressures

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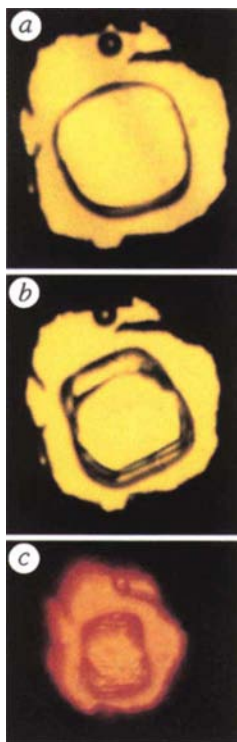
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SOLID hydrogen is predicted^{1,2} to become metallic at high pressures. Although metallization was recently reported in high-pressure shock-wave compression experiments using liquid hydrogen³, efforts to understand the high-pressure behaviour of the solid phase have relied mainly on spectroscopic studies in the diamond-anvil cell^{4–6} and on *ab initio* calculations^{7–10}. Central to these studies is the high-pressure crystal structure—something that is difficult to determine in the diamond-anvil cell—and its pressure dependence. Here we report X-ray diffraction measurements of the structure of single-crystal molecular hydrogen at pressures of up to 109 GPa for H₂ and 119 GPa for D₂. From these measurements we deduce the high-pressure equation of state (EOS: the volume–pressure relation). We find that solid hydrogen is more compressible than previously thought, that the crystal becomes increasingly anisotropic with pressure, and that the difference in EOS between H₂ and D₂ is unexpectedly small. The ‘softening’ of the EOS relative to previous estimates⁴ increases by about 25% the expected transition pressure to the atomic phase. Our EOS differs significantly from that predicted by *ab initio* calculations^{7,9}, indicating that theoretical understanding of the behaviour of dense hydrogen remains incomplete.

Measurements of the structural properties of hydrogen at high pressure by X-ray diffraction are of the utmost difficulty because of hydrogen’s small scattering efficiency, which can be many orders of magnitude weaker than the background. The problem of weak diffraction intensity is partially overcome by the use of the single-crystal X-ray technique, first carried out at 5.4 GPa (ref. 11). Measurements at higher pressures are complicated by the need for a significant reduction in the volume of the sample chamber (by five orders of magnitude from 5 GPa to 150 GPa) and by the fragmentation of the single crystals, which reduces the intensity of the reflections by up to three orders of magnitude when going from 5 to 50 GPa.

The problem of the drastic reduction in diffracted intensity for higher-pressure measurements can be overcome by employing the high brilliance of a synchrotron radiation source and a new experimental technique based on a combination of single-crystal X-ray methods with energy-dispersive diffraction¹². Diffraction measurements on H₂ up to 42 GPa have been reported previously^{4,13}. We have used the BL3 wiggler beam line at the European Synchrotron Radiation Facility (ESRF), the brightness of which decreases significantly the time needed to search for the diffraction peaks and to reduce the otherwise dominant background signal from the diamond anvils. The latter is made possible by the ‘lozenge geometry’ of fixed-angle energy-dispersive diffraction with narrow collimation of the incident and diffracted X-ray beams¹². The X-ray beam was collimated without focusing optics by micro-slits to $15 \times 15 \mu\text{m}$. Pressure was measured using the quasi-hydrostatic ruby scale¹⁴ (good hydrostaticity was observed up to the highest pressure). The accuracy of the pressure measurement was estimated to be $\Delta P = \pm 0.3 \text{ GPa}$ relative to the ruby scale. The error in the volume measurement was estimated to be $\pm 2 \times 10^{-3} \text{ cm}^3 \text{ mol}^{-1}$.

FIG. 1 Photomicrographs of the evolution with pressure of a single crystal of D_2 (centre of each figure) in helium. The single crystal of D_2 was grown from an initial mixture around 0.26 D_2 mole fraction in the D_2 -rich solid–He-rich fluid equilibrium at nearly 10 GPa (a). The pressure was then carefully increased over a few days to around 14 GPa (b) where the solid–solid equilibrium occurs. Slow increase of pressure up to 119 GPa (c) minimizes the breakup of the single crystal (the red colour is due to filters used in the ruby pressure-measurement system on the X-ray beamline). The difference between the measurements on pure solid hydrogen samples and those on solid hydrogen in helium is smaller than the variation expected for a possible 1 mol% He solubility in solid hydrogen ($\Delta V/V = -4 \times 10^{-3}$) and is undetectable within error. This, in addition to Raman studies of the phase diagram to 200 GPa and 80 K, indicates there is negligible solubility of He in solid hydrogen, at the conditions present in these experiments. A ruby ball (6 μm diameter), used for pressure measurement, is at the top centre. The sample chamber dimensions changed from $\sim 50 \mu\text{m}$ diameter, 10 μm thickness at 14 GPa to $\sim 36 \mu\text{m}$ diameter, 7 μm thickness at 119 GPa. Similar results were obtained for H_2 in He. For single-crystal X-ray diffraction experiments, the X-ray viewing angle of both sides of the sample needed to be kept as large as $\pm 40^\circ$. X-ray measurements above 100 GPa were made possible by the development of a new membrane X-ray cell with boron seats of half-sphere geometry. The cell was loaded in a high-pressure vessel at room temperature with high-purity gases.



The pressure range for diffraction from hydrogen was initially extended to 50 GPa. Above this pressure, however, the diffraction peaks merged into the strong background signal (primarily owing to Compton scattering from the diamond). We were able to supersede this limit by growing a single crystal of hydrogen in helium. The growth method is based on the nearly complete immiscibility of H_2 (or D_2) and He in the solid phase¹⁵ (Fig. 1). Solid helium is the most hydrostatic medium under these conditions and acts as a cushion around the hydrogen crystal, reducing the risk of fragmentation of the crystal on increasing pressure.

Three classes of diffraction peaks (100, 002 and 101) were followed to the maximum pressure. The reflections were related by the orientation matrix of the hexagonal close packed structure ($P6_3/mmc$, with rotating molecules on lattice sites) and their signal to noise ratio was still very good (>25) at 119 GPa. Altogether, seven single crystals were studied: three single crystals of pure H_2 limited to below 50 GPa, two single crystals of H_2 in helium and two single crystals of D_2 in helium. These crystals were grown in different orientations, with the c axis almost parallel or almost perpendicular to the faces of the diamond anvils. No effects of uniaxial strain were observed. The complete set of data measured at 300 K at the ESRF is plotted in Fig. 2. A Vinet form¹⁶ (Fig. 2 legend) was adjusted to represent the data of the $T = 300$ K EOS of H_2 within error bars (solid line in Fig. 2—the error bars are not much greater than the thickness of the line). This EOS is significantly more compressible than that previously constrained⁴ from lower-pressure data to 42 GPa (dashed line in Fig. 2). The difference amounts to 12% for the pressure and 20% for the compressibility at 120 GPa. In addition, the almost linear decrease of the c/a ratio of the hexagonal cell (Fig. 2, inset) indicates that the crystal becomes increasingly anisotropic with pressure. Freely rotating molecules give rise to a c/a close to the ideal value 1.633,

as observed below 10 GPa (ref. 4). We therefore interpret the observed deviation of c/a ratio from ideal value as a gradual effect of orientation of the H_2 molecular axis within phase I (ref. 4).

The near-universal equation of state of Vinet *et al.*¹⁶ has been shown to represent very well the compressional effects associated with electronic energy¹⁶. Its applicability for extrapolating the EOS of hydrogen to higher pressures could be questioned because of possible increasing contributions of zero-point motion. To examine this, we use the Mie–Grüneisen–Debye model¹³—determined from measurements of the optical phonon to pressures above 100 GPa—to subtract the thermal and zero-point pressures from our measured pressure. A Vinet function representing only the electronic contribution of the EOS was then fitted to these reduced data. Addition of the zero-point pressure ($\sim 6\%$ of the pressure above 100 GPa) to this function gives the $T = 0$ K equation of state of hydrogen (Fig. 3, thin line). We believe this pressure–volume equation of state adequately describes the compression of molecular hydrogen at higher pressure because the compression is largely completed between 5 and 120 GPa (Fig. 3, thick line) and hence the EOS is well constrained by experiment. We note also that the extrapolation of the EOS up to 350 GPa is almost independent of the analysis made, that is, whether the phonon contribution is taken into account explicitly (fit of reduced data) or implicitly (fit of measured data). This conclusion could be dependent on phase transitions in molecular hydrogen, but these are predicted to be largely orientational and weakly displacive with small volume discontinuity (for example the transition to phase III above 150 GPa; ref. 4). Figure 3 also shows that the volume determined from the EOS at 350 GPa is close to the most refined calculation published to date on atomic hydrogen⁸.

Dense hydrogen is perhaps the most fundamental many-body system, with all interactions described exactly by the bare Coulomb potential. It is also the most challenging for theory

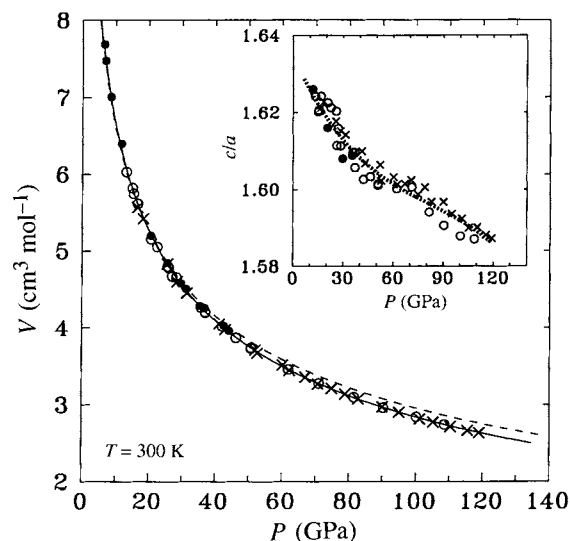


FIG. 2 Main figure, the 300 K pressure–volume equation of state of the hydrogen isotopes. The symbols indicate the ESRF measurements: solid circles, solid H_2 ; open circles, solid H_2 in helium; crosses, solid D_2 in helium. The solid line is a fit of the H_2 X-ray data with the Vinet function¹⁶:

$$P = 3K_0 \left(\frac{V}{V_0} \right)^{-\frac{2}{3}} \left[1 - \left(\frac{V}{V_0} \right)^{\frac{1}{3}} \right] \exp \left\{ \frac{3}{2} (K'_0 - 1) \left[1 - \left(\frac{V}{V_0} \right)^{\frac{1}{3}} \right] \right\}$$

with the adjusted parameters $V_0 = 25.433 \text{ cm}^3 \text{ mol}^{-1}$, $K_0 = 0.162 \text{ GPa}$ and $K'_0 = 6.813$. The dashed line is the EOS previously obtained from X-ray data at the NSLS^{4,12,13}. Inset, the evolution of the c/a ratio of the hexagonal cell with pressure: same symbols as the P – V data; the dashed line is drawn as a guide to the eye.

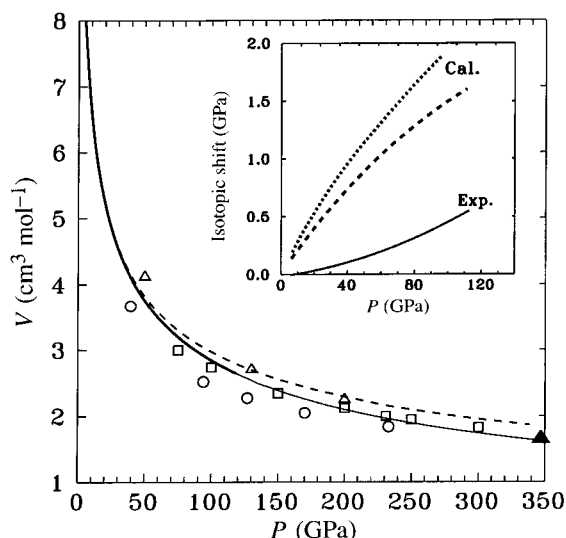


FIG. 3 Main figure, comparison between theoretical and experimental equations of state for H_2 . The solid line represents the experiment-based EOS at $T = 0 \text{ K}$: the portion of the line below $\sim 120 \text{ GPa}$ represents the temperature-reduced ($T = 0 \text{ K}$) X-ray data (the thermal pressure is $< 0.3 \text{ GPa}$ above 30 GPa); the portion of the line above $\sim 120 \text{ GPa}$ is given by the sum of the electronic contribution (Vinet function with parameters $V_0 = 15.690 \text{ cm}^3 \text{ mol}^{-1}$, $K_0 = 0.999 \text{ GPa}$ and $K'_0 = 5.799$) and the zero-point pressures are made with the Mie-Grüneisen-Debye model¹³. The symbols indicate various theoretical calculations discussed in the text: open squares, LDA molecular solid⁷; open triangles, QMC molecular solid⁹; open circles, molecular-dynamics simulations of an LDA fluid (1,000 K) (ref. 17); solid triangle, QMC atomic solid⁸; dashed line, calculation with the effective pair potential¹⁸. Inset, the difference in pressure between H_2 and D_2 at a given volume at 300 K: the lower dashed line is the calculation with the effective pair potential of ref. 18, the upper dotted line is the calculation with the Mie-Grüneisen-Debye model¹³, and the solid line is the experimental determination.

owing to the large zero-point motion and anharmonic effects of the protons. The results of two theoretical calculations are compared in Fig. 3 to the experiment-based EOS. Well converged calculations⁷, using the Local Density-functional Approximation (LDA) for treating the electronic exchange-correlation functional, give volumes within 6% of experimental value but disagree more in the compressibility. Also, this work predicts an isotopic shift of the order of 1.5 GPa at 120 GPa, larger than the experimental shift (Fig. 3, inset). This comparison indicates the need to treat electrons beyond the LDA approximation and protons beyond the harmonic approximation in dense hydrogen, as previously pointed out⁸. Quantum Monte Carlo (QMC) methods, which in principle accurately treat the quantum mechanics of interacting electrons and protons (the only essential approximation being the nodal surface of the electrons) give volumes⁹ 8% above the experimental values. This difference could be due to size effects or the admitted insufficient optimization of the simulations.

Two calculations were recently used to derive the sound velocity in the molecular layer of Jupiter. The EOS determined in these studies, within the LDA framework¹⁷, and within an effective pair-potential approach¹⁸, are compared to experiment in Fig. 3. The present results indicate that the sound velocity of hydrogen (given by $(dP/d\rho)^{1/2}$, where P is pressure and ρ is density) in the pressure range of this comparison is smaller than that estimated previously. This could be important in the refinement of models of the jovian interior and in the interpretation of possible global oscillation data for the planet^{17,18}. The first calculation, however, was done for the fluid phase at 1,000 K and the systematically smaller calculated volume—the discrepancy should even be larger if thermal pressure were subtracted—could also mean that fluid hydrogen becomes denser than the solid at very high pressures. If real, this would imply a negative P - T slope of the melting curve at these pressures, a property with possible implications for laser fusion applications and astrophysics. If the melting curve of hydrogen has a negative slope, the fluid phase has a wider stability domain and consequently the occurrence of solid molecular hydrogen in the interior of the gas-giant planets and of brown dwarfs is less probable. Moreover, a negative slope of the melting curve implies a wider P - T domain in which the Lawson criteria for fusion (temperature $\times$ density $\times$ confinement time $>$ constant) is satisfied. Hence, this could offer new routes to laser fusion, for example by starting at higher density.

The difference between the EOS of H_2 and D_2 is quantified by plotting (Fig. 3 inset) the difference between separate Vinet fits of H_2 and D_2 data at 300 K (in so doing, the error bars are expected to

be smaller than the sum of the error bars of the two measurements, $\pm 0.7 \text{ GPa}$). This isotopic shift in the EOS is seen to be significantly smaller than that calculated with an effective pair potential or with the Mie-Grüneisen-Debye model mentioned above. The explanation of this difference could originate from the dynamical coupling between electrons and protons⁸.

The softer EOS leads to enhanced stability of the molecular phase relative to the atomic metal phase. An estimate of this effect can be obtained by comparing the enthalpy of the molecular phase, calculated by integration of VdP (at zero pressure, it is essentially the ground-state energy of the hydrogen molecules), and that calculated theoretically for the diamond phase of atomic hydrogen, predicted to be stable in a fully optimized QMC study⁸. With the EOS determined from the lower-pressure X-ray data^{4,13}, a transition pressure of $\sim 500 \text{ GPa}$ is obtained, whereas with the present EOS the transition is $\sim 620 \text{ GPa}$. Although this indicates that the atomic metal phase is beyond the range of current static-pressure techniques, it also supports predictions¹⁹ that metallization should take place in the molecular phase (for example by band overlap) at lower pressure before dissociation⁴. Theoretical calculations suggest that the pressure of molecular metallization hinges upon molecular orientational ordering and crystal structure. In this respect, the determination of the structure of phase III above 150 GPa is an important issue which is now within reach with these techniques. □

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Extraterrestrial ^3He as a tracer of marine sediment transport and accumulation

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THE deposition rate of deep-sea sediments, and their focused redeposition by deep-sea currents, can be evaluated from analyses of sedimentary ^{230}Th with a temporal resolution limited only by bioturbation^{6,7,10,11}. ^{230}Th is produced uniformly throughout the ocean by radioactive decay of dissolved ^{234}U and is removed sufficiently fast by sorption onto sinking particles to act as a 'constant-flux' tracer of sedimentation rates. But the half-life of ^{230}Th (75 kyr) limits its use for this purpose to the past 200–250 kyr. Here we explore the use of extraterrestrial ^3He from interplanetary dust particles^{1–4} (IDPs) as a constant-flux proxy that is free from this limitation. A comparison of ^3He with ^{230}Th in two cores from the equatorial Pacific Ocean indicates that the variability in the mean flux of IDPs over the past 200 kyr is less than 75%. But in contrast to this relatively constant rate of supply of ^3He to the deep sea, the local burial rates of ^3He and ^{230}Th have varied by a factor of five over the past 450 and 200 kyr, respectively. We interpret this variability as reflecting sediment focusing, with a temporal pattern that suggests regular cycles of climate-driven reorganization of near-bottom currents in the deep Pacific Ocean.

The residence time of dissolved ^{230}Th in the ocean with respect to sorption by marine particulate phases is sufficiently short that, to a first approximation, the rain rate of particulate ^{230}Th sinking to the sea bed, F_{Th} , is equivalent to the integrated rate of ^{230}Th production by U decay in the overlying water column^{5,6}, P_{Th} . Sediment trap data confirm that even with 15-fold variations in total mass flux of particles, F_{Th} deviates from P_{Th} by less than 40% (refs 7–9). Because P_{Th} is constant, the unsupported or excess Th concentration in sediments ($x_{\text{S}}^{230}\text{Th}$) is inversely related to the rain rate of particulate matter^{5,6}, F_{m} :

$$x_{\text{S}}^{230}\text{Th} = P_{\text{Th}}/F_{\text{m}} \quad (1)$$

where $x_{\text{S}}^{230}\text{Th}$ is in units of d.p.m. g⁻¹, P_{Th} is in d.p.m. cm⁻² kyr⁻¹ and F_{m} is in g cm⁻² kyr⁻¹. Equating the rain rate of ^{230}Th with its production rate permits the evaluation of rain rates for other sedimentary components before modification by lateral advection as:

$$F_i = \frac{C_i \beta z}{x_{\text{S}}^{230}\text{Th}} \quad (2)$$

where F_i is the rain rate of particulate component i , C_i is the concentration of i in the sediments, $\beta z = P_{\text{Th}}$ where $\beta = 2.65 \times 10^{-5}$ d.p.m. cm⁻³ kyr⁻¹ and z is the depth of the water column in metres. The superscript 'o' following $x_{\text{S}}^{230}\text{Th}$ indicates decay correction of $x_{\text{S}}^{230}\text{Th}$ activity to the time of deposition which, in turn, requires an independent chronology, usually provided by ^{18}O stratigraphy. Furthermore, because the burial rate of $x_{\text{S}}^{230}\text{Th}$ is equivalent to its rain rate, as further modified by lateral advection of sediment, comparing the burial rate of ^{230}Th with P_{Th} enables the evaluation of focused deposition of sediments by deep-sea currents^{7,10,11}. This comparison also allows the evalua-

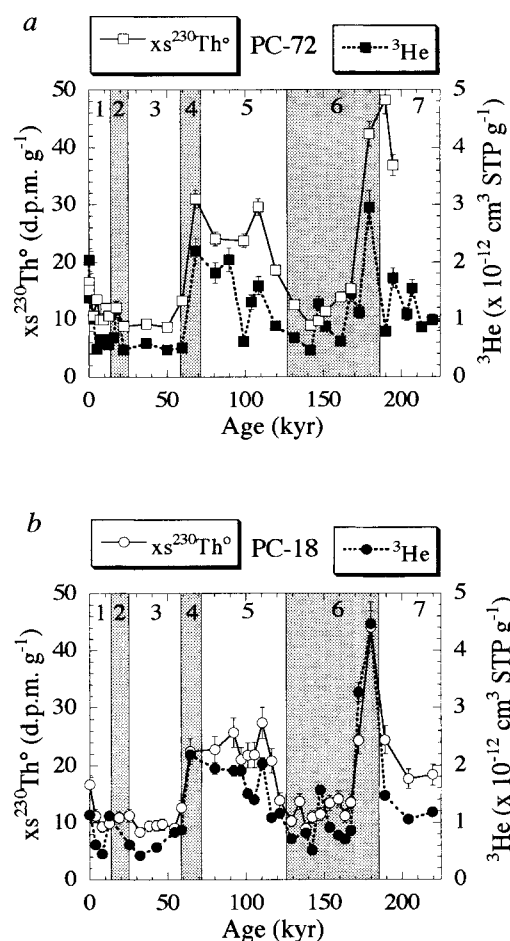


FIG. 1 Down-core age profiles of $x_{\text{S}}^{230}\text{Th}$ activities and ^3He concentrations for PC-72 (a) and PC-18 (b). Age boundaries for glacial-interglacial stages are from Imbrie et al.¹². The results for ^3He concentrations, $^3\text{He}/^4\text{He}$ ratios, and excess initial ^{230}Th activities in the first 220 kyr of sediment from PC-18, and of sediment ranging in age from 200–450 kyr from PC-72, are available (see Supplementary Information). Tabulated results for the first 200 kyr of PC-72 are presented elsewhere³.

tion of changing patterns of sediment focusing in response to reorganization of ocean circulation.

Samples from two piston cores (TT013-PC-18, 2° S, 139° W, 4,354 m water depth; TT013-PC-72, 0°, 139° W, 4,298 m water depth) recovered during the US Joint Global Ocean Flux Study (JGOFS) Equatorial Pacific Process Study (EqPac) have been analysed for helium, uranium and thorium isotopes. Sediment for both cores consists predominantly of CaCO_3 with minor opal, organic, terrigenous and IDP components. The age model for each core is tied to the SPECMAP¹² stacked $\delta^{18}\text{O}$ record (a detailed age model for PC-72 is presented in ref. 13).

Down-core concentration profiles of ^3He and $x_{\text{S}}^{230}\text{Th}$ exhibit remarkable similarity (Fig. 1) and regular patterns in both cores. Maximum concentrations of both tracers are found in late interglacial to earliest glacial-age sediments, after which concentrations drop abruptly—for example in early glacial stage 6 and in stage 4. This regular pattern in the concentration profiles of two tracers of very different origin, extraterrestrial ^3He from IDPs and radiogenic $x_{\text{S}}^{230}\text{Th}$ produced by U decay in the water column, reflects the common effect of variable dilution by the principal sedimentary components (for example, CaCO_3) which, for ^{230}Th , is expressed quantitatively by equation (1). Although CaCO_3 is the dominant component (75–95% by weight) throughout these cores, it has long been known that equatorial Pacific sediments are characterized by regular patterns of variable carbonate

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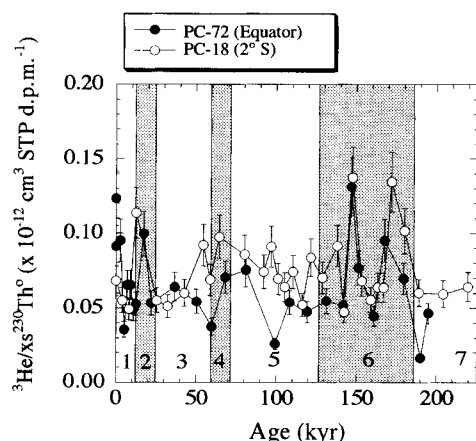


FIG. 2 $^3\text{He}/x\text{s}^{230}\text{Th}^0$ ratios versus age for both cores. (Age boundaries for glacial-interglacial stages are from Imbrie et al.¹²).

accumulation, with maximum accumulation during glacial periods and minimum during interglacials¹⁴. Furthermore, it has been established that the Pacific carbonate cycles lag the global ice volume record, upon which the isotopic stages in Fig. 1 are based, by roughly 10 kyr (refs 15–18). The effects of variable carbonate accumulation are clearly manifest in the concentration profiles of He and Th, even to the level of detail that the 10-kyr lag relative to global ice volume is evident, in that isotope concentrations do not drop until well into glacial stages 6 and 4 (Fig. 1).

Both the mean fallout rate of IDPs and its variability can be constrained by comparing the $^3\text{He}/x\text{s}^{230}\text{Th}^0$ ratios through time. Records from both cores (Fig. 2) exhibit high-frequency variations that appear to be synchronous but without a systematic relationship to climate cycles. The average $^3\text{He}/x\text{s}^{230}\text{Th}^0$ ratio for both cores is $0.070 \times 10^{-12} \text{ cm}^3 \text{ STP d.p.m.}^{-1}$ and has a maximum variability of 73% at the 2σ confidence interval, one-quarter of which may be due to analytical uncertainty. The mean flux of ^3He derived from the Th-profiling method (equation (2)) is $(7.8 \pm 2.3) \times 10^{-13} \text{ cm}^3 \text{ STP cm}^{-2} \text{ kyr}^{-1}$, equivalent, within error, to previously estimated values^{1–3}. Such a flux of extraterrestrial ^3He corresponds to a mean global flux of IDPs (which retain their extraterrestrial ^3He) of $(2.1 \pm 0.6) \times 10^{11} \text{ g kyr}^{-1}$, assuming a ^3He content for pure IDPs^{19,20} of $1.9 \times 10^{-5} \text{ cm}^3 \text{ STP g}^{-1}$. Our estimated mean global flux of helium-retentive IDPs represents about 0.5% of the total extraterrestrial dust flux²¹.

The small variability in $^3\text{He}/x\text{s}^{230}\text{Th}^0$ ratio has important implications for the recent hypothesis linking the 100-kyr climate cycle of global ice volume to the 100-kyr periodicity in the inclination of the Earth's orbit²². Muller and MacDonald²² predicted that the accretion rate of cosmic dust to the Earth varies with the 100-kyr cycle of inclination and that this, in turn, may be responsible for forcing the 100-kyr climate signal observed in the $\delta^{18}\text{O}$ ice volume record. In contrast to an earlier study of North Atlantic sediments⁴, we find no evidence from the ^3He record in the equatorial Pacific for a 100-kyr periodicity in the accretion rate of IDPs. Indeed, a major implication of our $^3\text{He}/x\text{s}^{230}\text{Th}^0$ data is that the accretion rate, or supply from space, of helium-retentive IDPs is virtually constant. More work is needed to test the origin of the high-frequency variability in the He/Th ratios; however, a boundary scavenging effect²³ can be ruled out, as down-core profiles of $^{231}\text{Pa}/^{230}\text{Th}$ ratios in both cores show no coherent relationship to the variability of the $^3\text{He}/^{230}\text{Th}$ ratios (R.F.A., unpublished results).

Concentrating on the longer record of PC-72, we find a regular pattern in the accumulation (or burial) of ^3He and $x\text{s}^{230}\text{Th}^0$ that indicates a coherent relationship between sediment focusing and the carbonate cycles. For the entire 450-kyr period, maximum concentrations of ^3He (Fig. 3c)—found in late interglacial to

earliest glacial-age sediments (from $\delta^{18}\text{O}$ record; Fig. 3a)—correlate well with minimum CaCO_3 concentrations (Fig. 3b), reflecting the dilution of a constant rain of IDPs by variable accumulation of CaCO_3 . A consistent pattern of $x\text{s}^{230}\text{Th}^0$ concentration is observed in the latter half of the record (Fig. 3c). Burial rates for both $x\text{s}^{230}\text{Th}^0$ and ^3He are calculated by multiplying the $x\text{s}^{230}\text{Th}^0$ and ^3He concentrations by the corresponding ^{18}O -derived bulk sediment accumulation rates¹³. In contrast to the constant rain rates of both nuclides, the burial rates exhibit synchronous five-fold variations over the past 200 kyr. Identical variations occur in PC-18. A five-fold change in the burial rate of $x\text{s}^{230}\text{Th}^0$ exceeds by more than an order of magnitude any change that can be attributed to variability of particle scavenging intensity^{7–9}. Similarly, the five-fold range in ^3He accumulation exceeds by far the maximum variability of IDP accretion rate ($<75\%$) permitted by the $^3\text{He}/x\text{s}^{230}\text{Th}^0$ ratios (Fig. 2). The pronounced variability of the burial rates of $x\text{s}^{230}\text{Th}^0$ and of ^3He is interpreted, therefore, to reflect a local reorganization of sediment focusing (that is, lateral advection), and of the deep currents responsible for particle transport. Although Farley and Patterson⁴, in their study of North Atlantic sediments, mention the possibility that the observed ^3He variations are due to particle focusing, they prefer

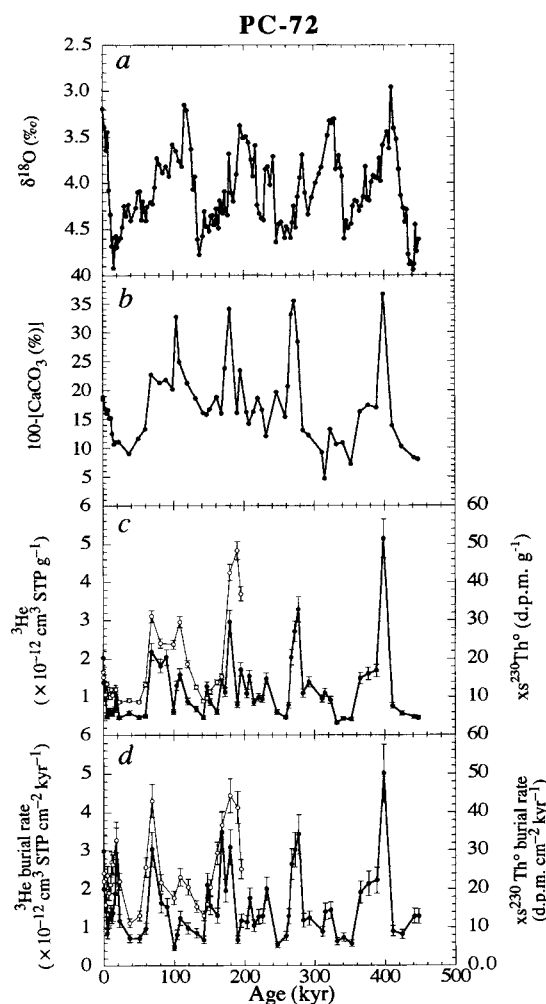


FIG. 3 Down-core profiles, plotted against age, for PC-72: a, $\delta^{18}\text{O}$ of benthic foraminifera *Cibicides wuellerstorfi* (adjusted by 0.64‰ to allow for the systematic offset from equilibrium with bottom waters in the formation of *Cibicides*'s shells); b, calcium carbonate content—measured by standard coulometric procedures at Lamont-Doherty Earth Observatory Core Lab—presented as $100 - [\text{CaCO}_3] (\%)$ to orient peaks with the other records; c, ^3He concentrations (filled circles) and $x\text{s}^{230}\text{Th}^0$ activities (open circles); and d, ^3He (filled circles) and $x\text{s}^{230}\text{Th}^0$ (open circles) burial rates.

an interpretation in which cyclicity in cosmic-dust accretion rate is the cause of ^3He variability.

The fact that the inventory of xsTh , integrated over the past 200 kyr, exceeds that expected from local production (P_{Th}) by a factor of two demonstrates that extensive sediment focusing occurs, on average, in this region of the equatorial Pacific. More interesting that the average focusing factor, however, is the regular pattern of variable sediment focusing. Periods of maximum sediment focusing (maximum burial rates of ^3He) correspond to periods of minimum carbonate concentration (maximum dissolution) during the last four glacial-interglacial cycles. Both processes occur regularly during the middle part of the growth of continental ice sheets (increasing $\delta^{18}\text{O}$; compare Fig. 3a and d). Although the nature of the reorganization of deep-sea currents responsible for the alternating pattern of sediment focusing remains undetermined, the correlation between deep Pacific circulation and sedimentary cycles of carbonate accumulation provides evidence for a linkage—over glacial-interglacial times—between the reorganization of ocean circulation and the ocean-atmosphere carbon cycle^{24,25}. □

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Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia

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STROKE is the third leading cause of death, and vascular dementia the second cause of dementia after Alzheimer's disease. CADASIL (for cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) causes a type of stroke and dementia whose key features include recurrent subcortical ischaemic events and vascular dementia and which is associated with diffuse white-matter abnormalities on neuro-imaging^{1,2}. Pathological examination reveals multiple small, deep cerebral infarcts, a leukoencephalopathy, and a non-atherosclerotic, non-amyloid angiopathy involving mainly the small cerebral

arteries³. Severe alterations of vascular smooth-muscle cells are evident on ultrastructural analysis⁴. We have previously mapped the mutant gene to chromosome 19 (ref. 5). Here we report the characterization of the human *Notch3* gene which we mapped to the CADASIL critical region. We have identified mutations in CADASIL patients that cause serious disruption of this gene, indicating that *Notch3* could be the defective protein in CADASIL patients.

CADASIL was originally mapped to chromosome 19 in two families⁵. Subsequent linkage analysis of 13 additional pedigrees led us to establish the genetic homogeneity of this condition and to refine the locus assignment to within a 2-centimorgan (cM) interval bracketed by *D19S199* and *D19S226* (ref. 6). Analysis of additional pedigrees with new microsatellite markers allowed us to map the centromeric boundary at *D19S253* and the telomeric one at *D19S929*. Interestingly, the meiotic recombinant defining the *D19S929* boundary was observed in an asymptomatic 40-year-old patient with an inconclusive result from cerebral magnetic resonance imaging, but whose skin biopsy showed typical alterations in vascular smooth-muscle cells⁴.

We constructed YAC and BAC contigs encompassing the critical region, whose size was estimated to be ~800 kilobases (kb) (Fig. 1). There was a high density of *NotI*, *EagI* and *SacII* restriction enzyme digestion sites, suggesting the presence of numerous genes (data not shown). Of the candidate transcripts identified through direct selection of complementary DNA^{7,8}, 417Nc5 showed strong homology with the coding 5' end of the mouse *Notch3* gene⁹. Further evidence that a *Notch3* human homologue could be located within this region was provided by previous fluorescence *in situ* hybridization (FISH) mapping on chromosome 19p 13.1–13.2 of a human cosmid shown to cross-hybridize with mouse *Notch3* cDNA¹⁰. We assigned 417Nc5 and three overlapping mouse *Notch3* cDNA fragments (X20, Mel3 and JJ5) to the CADASIL critical region, between *14G5L* and *D19S841* (Fig. 1). In *Drosophila*, *Notch* encodes a glycosylated transmembrane receptor of relative molecular mass 350K, which is involved in cell-fate specification during the development^{11,12}. Several *Notch* homologues have been identified in vertebrates¹⁰; two of which (*Notch1*, also called *TAN1*, and *Notch2*) were characterized in man^{13,14}. Their overall structure and sequences

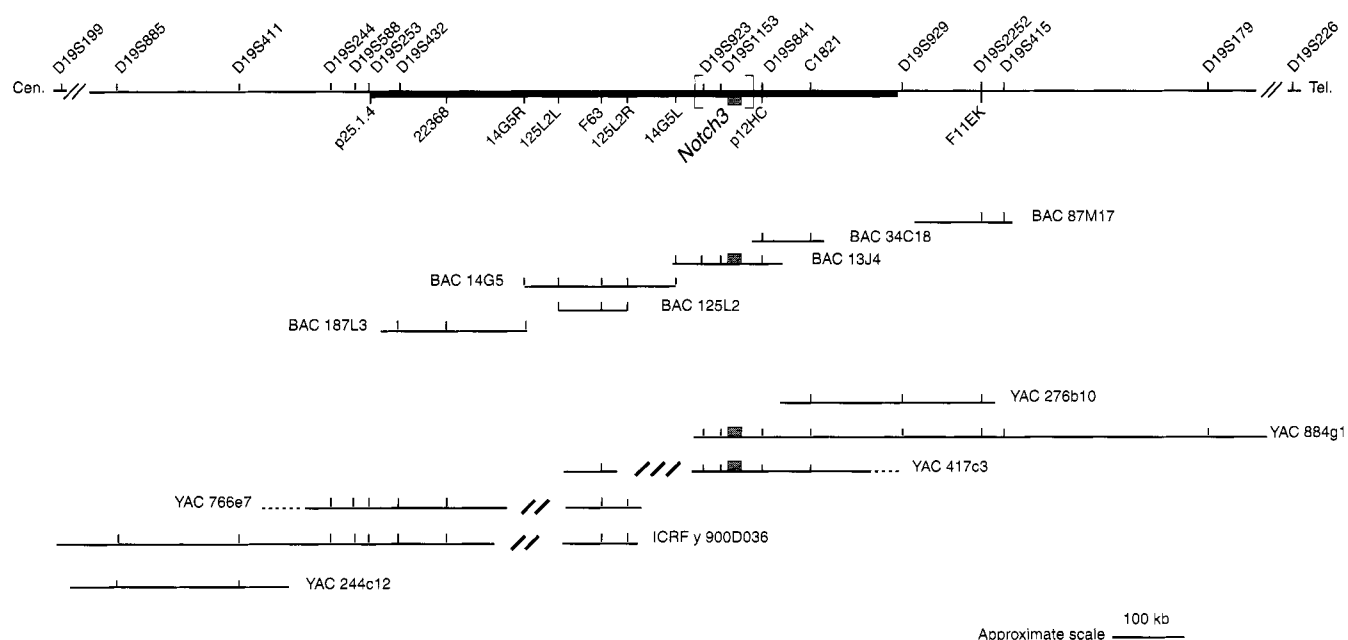


FIG. 1 Genetic and physical map of the CADASIL region, showing the location of the *Notch3* gene. Human chromosome 19 is shown as a horizontal line. The CADASIL critical region, as defined by two obligate recombination events at D19S253 and D19S929, is depicted as a closed bar. Microsatellite markers and single-copy probes are represented respectively above and below the line. Markers between brackets could not be accurately positioned with respect to each other. BAC and YAC

clones are represented by horizontal bars drawn to scale. A minimal set of six overlapping YAC clones, covering a 1.8-Mb contig from D19S885 to D19S179 is represented. Chimaerism of YAC clones is depicted by a dotted line and interstitial deletions by slashes. Overlap of BAC clones was established by cross-hybridization with right (14G5R, 125L2R) and left BAC ends (125L2L, 14GL). The location of *Notch3* is shown as a shaded box. Cen, centromere; Tel, telomere.

share striking similarity with the *Drosophila Notch* gene. Although these genes are expressed in both embryonic and adult tissues^{9,13,15}, their functions in the latter remain largely unknown. Several proteins involved in the Lin-12/Notch signalling pathway have been identified in various species¹⁶⁻²¹. There is a remarkable conservation between a gene involved in the Lin-12/Notch signalling pathway (*Sel-12*)²² and one involved in early-onset familial Alzheimer's dementia (*S182*)²³. Although, to our knowledge, no clue exists at present as to the pathways that lead from *S182* mutations to the Alzheimer's phenotype, the striking homology of *S182* and *Sel-12* suggests that an abnormality in Notch signalling may be involved in the pathogenesis of this adult-onset condition. We therefore considered that *Notch3* could be involved in the pathophysiological mechanisms of CADASIL, another adult-onset neurological condition. On the basis of the strong sequence similarity of 417Nc5 with the mouse *Notch3* gene, we used mouse cDNA probes to isolate human *Notch3* cDNA and genomic

clones, the sequences of which were aligned to the mouse cDNA sequence; additional sequence data were obtained from a cDNA fragment (884Na4) and two genomic fragments (J431NH and J432NH), which we identified through cDNA selection on YAC 884g1 and characterization of *NotI-HindIII* subclones from BAC 13J4. Screening of the expressed-sequence-tags database (dBest) with all of these sequences led to the identification of additional cDNA clones. A contiguous stretch of 5,615 base pairs (bp) of human *Notch3* coding sequence, highly homologous to its murine coding counterpart (corresponding to nucleotides 261–5,876 of mouse *Notch3* cDNA; accession number X74760) was assembled (Fig. 2). Human and mouse proteins are 90% identical along the available overall sequence. Based on sequence alignment with the various functional domains identified in the mouse protein, this partial cDNA contig is predicted to contain 33 EGF domains, three Notch/Lin-12 repeats, as well as three Cdc10 ankyrin-like repeats. Northern blot hybridization with p28-20, a

human 1.45-kb partial cDNA probe that corresponds to the mouse EGF-like domains 10–22, revealed a unique and ubiquitous transcript in fetal as well as adult human tissues, whose size of 7.5–9.5 kb is similar to that of the mouse transcript⁹ (data not shown).

No gross rearrangement of genomic DNA was detected in CADASIL patients using various *Notch3* probes and enzymes combinations. A panel of 58 unrelated patients was then checked for point mutations. Alignment of the available genomic sequence to the cDNA contig identified at least 29 exons. Fourteen of these, for which both flanking intron sequences were available, were screened for mutations using single-strand conformation polymorphism (SSCP). These 14 exons

TABLE 1 *Notch3* mutations observed in unrelated CADASIL patients

Patient's identification	Nucleotide sequence alteration	Amino-acid change	Exons*	Frequency in control chromosomes
P56	TGG → TGT	W → C	N2 (EGF 1)	0/200
F21/F6/P36	CGC → TGC	R → C	N3 (EGF 4)	0/200
F1/P47	CGC → TGC	R → C	N3 (EGF 4)	0/200
P21	GCG → GCG	G → A	N5 (EGF 7)	0/134
P54	TGT → TAT	C → Y	N9 (EGF 13)	0/200
P18/P17	CGC → TGC	R → C	N9 (EGF 14)	0/200
P31	CGC → TGC	R → C	N9 (EGF 14)	0/200
P43	CGC → TGC	R → C	N12 (EGF 18)	0/200
P58	TGC → CGC	C → R	N20 (EGF 32)	0/200
F18	GCT → ACT	A → T	N25 (Cdc10 2)	0/200

* Murine functional homologue domains are indicated in brackets.

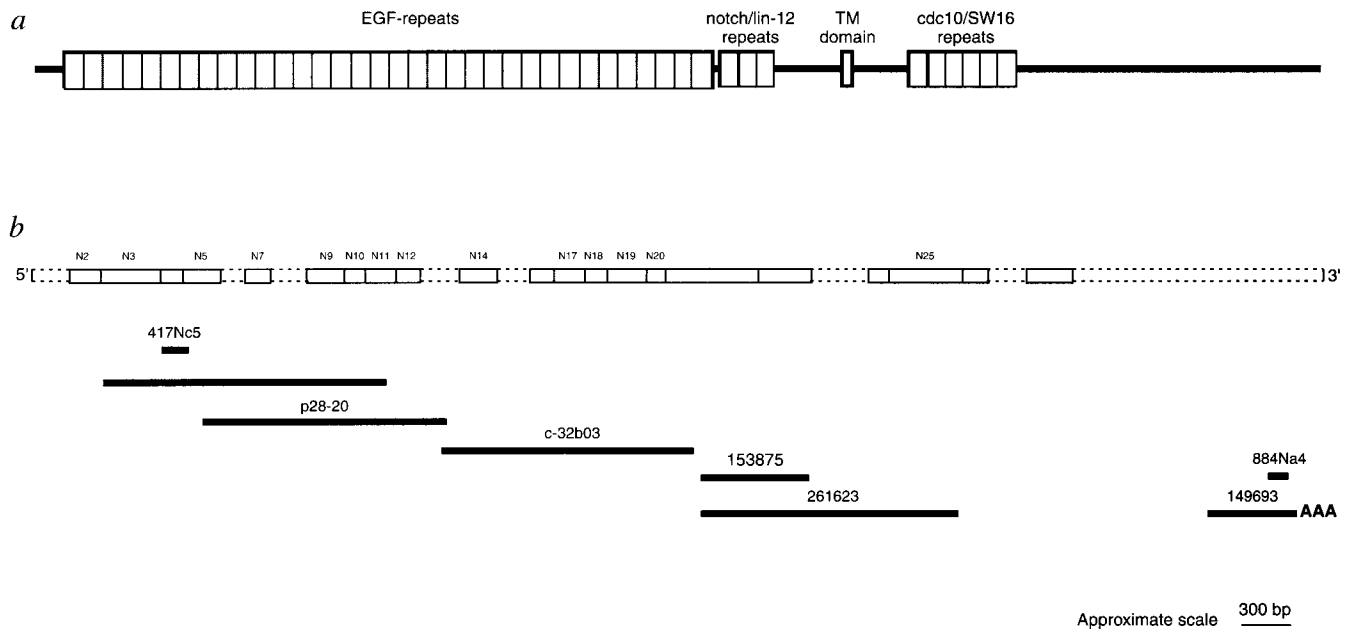


FIG. 2 Alignment of human partial cDNA clones with mouse *Notch3* and the exon-intron boundaries identified in human *Notch3*. *a*, Mouse *Notch3* gene. The 34 EGF-like domains, 3 *notch/lin12* and 6 *cdc10* repeats, as well as the transmembrane (TM) domain are indicated. *b*, Bottom: eight overlapping distinct cDNA clones were characterized; one of them,

149693, contained a poly(A) tail. Top: alignment of the available genomic sequence with the cDNA sequence data identified at least 29 exons. Filled boxes indicate exons for which both intron boundaries have been determined; dotted lines indicate a lack of available data on genomic structure. Exons that have been screened for mutations are numbered.

include 2,773 bp of coding sequence. There were 11 conformational variants within 7 exons, 10 of which were observed in 14 unrelated patients and none of the 200 control chromosomes. They were shown by sequencing to be due to nucleotide substitutions resulting in an amino-acid change. Co-segregation of the abnormal conformer with the affected phenotype was established in the six pedigrees available in this set of 14 patients. The eleventh variant was seen in patients and in controls, and sequencing showed that it was due to a silent nucleotide change. Nine of the ten mutations lay in seven distinct EGF-like domains, the last one being located within the highly conserved *cdc10*-repeat domain (Fig. 3). Within the EGF-like domains, two mutations should alter a cysteine residue, six others replace a conserved residue with an additional cysteine, and one replaces a highly conserved glycine with an alanine (Table 1). All these missense mutations may result in severe disruption of the Notch3 protein, as suggested by the highly conserved nature of the amino-acid residues involved, particularly the cysteines that are key features of EGF-like domains²⁴. These results indicate that these nucleotide substitutions are pathogenic mutations rather than rare polymorphisms.

The involvement of a member of the *Notch* gene family in an adult-onset neurological disorder raises the question of how *Notch3* mutations can lead to the CADASIL phenotype. *Notch* is known for its role in specifying cell fate during *Drosophila* development. The only human disorder implicating a *Notch*

gene is an adult T-cell leukaemia, which is associated with a truncation of the *TAN-1* (*Notch1*) transcript¹³. CADASIL is not associated with any developmental abnormality in affected families or with any neoplasia. On the basis of an analysis of *Drosophila* mutants it has been proposed that Notch may be a



FIG. 3 Predicted amino-acid sequence of the 7 exons (N2, N3, N5, N9, N12, N20, N25) with mutations in CADASIL patients indicated by arrowheads. Human and mouse sequences are aligned; identical residues are indicated by asterisks. The numbering of human exons is indicated on the left, with homologous murine domains in brackets. Only partial sequence data are presented for exons N3 and N25.

receptor with different functional domains, with the intracellular domain having the intrinsic signal-transducing activity of the intact protein and the extracellular domain a regulating activity²⁵. Mutations in these various domains result in different phenotypic alterations in *Drosophila*^{25,26}. Interestingly, we did not notice any difference in the phenotypes of the CADASIL patients who carry mutations in EGF-like and Cdc10 domains, nor in the phenotypes of patients carrying mutations in distinct EGF domains. Further genotype/phenotype correlation studies should help to establish the function of this protein. Study of the expression of this protein and of the Notch signalling pathway in adult vascular and brain tissues will also help in understanding molecular mechanisms leading to the development of CADASIL and other cerebro-vascular diseases and dementia. The involvement of *Notch3* in CADASIL suggests that disruption of the Notch signalling pathway may be a key factor in adult-onset conditions causing dementia. □

Methods

Patients. We analysed a panel of 58 unrelated patients fulfilling clinical and neuroradiological criteria for CADASIL^{2,6}. 28 of these belong to previously linked families (ref. 6, and our unpublished results); family members of the remaining patients were not available for linkage analysis. Genotyping has been described⁶. Sequences of microsatellite markers are available through the Genome Data Base (Johns Hopkins University, Baltimore).

Physical map of the CADASIL region. YAC libraries from CEPH²⁷ were screened by PCR with microsatellite markers *D19S885*, *D19S253*, *D19S923* and *D19S929*. YAC and BAC libraries from ICRF and Research Genetics Inc. (ref. 96055) were screened by hybridization using single-copy probes p25.1.4 (1.4-kb *EcoRI* subclone of p13.1.25; ref. 28), IMAGE cDNA clone 22368 (ref. 29), F63 (0.6-kb *RsaI* fragment from a fetal brain cDNA clone isolated using primers of EST00679), p12HC (2-kb *HincII* fragment from cosmid 12909; ref. 30), F11EK (0.8-kb fragment from cosmid 11547; ref. 30). Subcloned BAC ends were isolated by hybridization of specific oligonucleotides from the left BAC end (CTC TAC AGT CGA CCT GCA GGC ATG CA) and from the right BAC end (GAT TAC GCC AAG CTA TTT AGG TGA CAC TAT AGA ATA C) to replicate filters of, respectively, Bluescript ad Puc 18 *EcoRI* libraries from each BAC. Contig assembly was established by STS and single-copy probes content. Chimaerism of YAC clones was analysed by FISH.

Identification of transcribed sequences: (1) CpG island characterization. *NotI*-*HindIII* libraries from BACs 13J4, 14G5 and 187L3 were constructed. Subclones were sequenced using vector primers and unique probes, prepared from these *NotI*-containing fragments, hybridized to zoo blots and northern blots. **(2) Direct cDNA selection.** Random-primed, reverse-transcribed cDNA pools from lymphoblastoid cells, fetal and adult brain were hybridized on YAC clones 766e7, 417c3 and 884g1, according to ref. 7. PCR-amplified cDNAs from a fetal brain cDNA library (Clontech) were hybridized on YAC clone 417c3, according to ref. 8. Physical mapping of selected clones was established by hybridization to *EcoRI*-digested YAC and *HindIII*-digested BAC southern blots.

Characterization of the human *Notch3* gene: (1) cDNA isolation. A total of 8 cDNAs were characterized. P28-20 and CNA202 were isolated from a human fetal brain cDNA library (Clontech, ref. HL3003a) by conventional hybridization procedures using two probes: (1) X20, a 4.1-kb mouse *Notch3* cDNA fragment, kindly provided by U. Lendahl and M. Lardelli, and (2) a 0.2-kb genomic fragment corresponding to a human *Notch3* exon N3 (see below). IMAGE cDNA clones²⁹ (149693, 153875, 261623) and Genexpress (c-32b03) cDNA clones were identified by computer screening the dBest database with a Blast-N algorithm, using sequences of, respectively, 884Na4, J432NH and J431NH fragments. **(2) Determination of intron-exon boundaries.** Replicates of a *Sau3A* library from BAC 13J4 were hybridized with three partial mouse *Notch3* cDNA fragments covering the whole cDNA sequence (X20, MeI3 and JJ5). Filters were hybridized in 50% formamide hybridization buffer and washed at 55 °C in 0.2× SSPE, 0.1% SDS buffer. Positive clones were sequenced using vector primers and sequences were aligned with human and mouse *Notch3* cDNA sequences. Double-strand sequencing of genomic DNA or cloned cDNA fragments was done on an ABI 377 DNA sequencer (Applied Biosystems) using dideoxy terminators. **(3) Expression analysis.** Northern blots containing human poly(A)⁺ RNAs from various adult and fetal tissues were purchased from Clontech (ref. 7760-1, 7756-1). Membranes were hybridized according to the manufacturer's instructions, washed at a final stringency of 0.2× SSPE, 0.1% SDS at 50 °C and exposed for 5 d at -80 °C for autoradiography.

Screening for mutations. Oligonucleotides were designed from intron-exon boundaries sequences to amplify genomic fragments of ~200 bp. Reactions were performed in a final volume of 25 µl, containing 100 ng genomic DNA, 0.5 µM of each primer, 150 µM dNTP mix, 1 × PCR *Taq* polymerase buffer (Cetus), 1 U of *Taq* polymerase (BRL), 1.5 µCi dCTP for 30 cycles of 94 °C for 15 s, 65 °C for 15 s, 72 °C for 15 s. PCR products were denatured in 50%

formamide and electrophoresed through a 6% non-denaturing polyacrylamide gel. DNA from CADASIL patients showing an altered SSCP band was directly sequenced after PCR amplification.

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Increased amyloid-β42(43) in brains of mice expressing mutant presenilin 1

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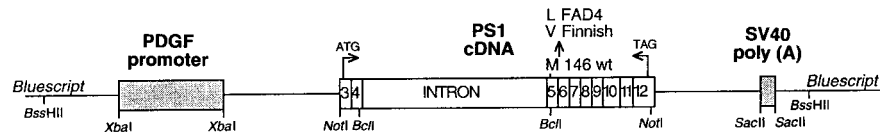
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MUTATIONS in the genes encoding amyloid-β precursor protein (APP)¹, presenilin 1 (PS1)² and presenilin 2 (PS2)^{3,4} are known to cause early-onset, autosomal dominant Alzheimer's disease. Studies of plasma and fibroblasts from subjects with these mutations have established that they all alter amyloid β-protein (βAPP) processing, which normally leads to the secretion of amyloid-β protein (relative molecular mass 4,000; M_r 4K; ~90%

FIG. 1 Constructs for the expression of wild-type and mutant *PS1* in transgenic mice.



A β 1–40, ~10% A β 1–42(43)), so that the extracellular concentration of A β 42(43) is increased⁵. This increase in A β 42(43) is believed to be the critical change that initiates Alzheimer's disease pathogenesis because A β 42(43) is deposited early and selectively in the senile plaques that are observed in the brains of patients with all forms of the disease. To establish that the presenilin mutations increase the amount of A β 42(43) in the brain and to test whether presenilin mutations act as true (gain of function) dominants, we have now constructed mice expressing wild-type and mutant presenilin genes. Analysis of these mice showed that overexpression of mutant, but not wild-type, *PS1* selectively increases brain A β 42(43). These results indicate that the presenilin mutations probably cause Alzheimer's disease through a gain of deleterious function that increases the amount of A β 42(43) in the brain.

Mice that were transgenic for wild-type or mutant *PS1* genes were generated as described (see Methods and Fig. 1). Founders were assessed by sequential Southern blotting of tail DNA, northern blotting of brain RNA and western blotting of brain proteins, to determine which lines to expand and use for experimentation. Mice expressing wild-type *PS1* (4 lines), the mutation in which methionine was substituted with leucine at residue 146 (M146L)² (2 lines) or valine, the M146V mutation^{6,7} (also 2 lines) were chosen for further analysis on the basis of their high protein levels. F₀ (3 wild-type lines) or F₁ (1 wild-type and the four mutant lines) mice were killed and their brains taken for analysis of *PS1* expression by northern blot (Fig. 2) and western blot (Fig. 3). These results showed that *PS1* messenger RNA was expressed in the transgenic mice, that the transgene was correctly spliced, and that this expression resulted in increased amounts of the ~46K *PS1* holoprotein and the amino-terminal ~27K *PS1* derivative, which have previously been described⁸.

The brains of mice from each line were homogenized in 70% formic acid and analysed with sandwich enzyme-linked immunosorbent assays (ELISAs) specific for A β 40 and A β 42(43) as described previously (Table 1)^{9,10}. Expression of mutant or wild-type *PS1* had no significant effect on brain A β 40, whereas expression of mutant but not wild-type *PS1* increased brain A β 42(43) (Table 1). In six non-transgenic mice, A β 42(43) concentration was 0.36 ± 0.06 pmol per g wet tissue, and in seven mice expressing wild-type *PS1* (the three founders and four F₁ mice from one of the wild-type lines), A β 42(43) concentration was 0.50 ± 0.07 pmol per g wet tissue. In five mice expressing the *PS1* M146L mutant (one founder and four F₁ mice), A β 42(43) was increased to 0.84 ± 0.09 pmol per g wet tissue, and in nine mice expressing the *PS1* M146V mutant (four F₁ mice from one line and 5 F₁ mice from the other), A β 42(43) was increased to 0.81 ± 0.04 pmol per g wet tissue. Analysis of the six nontransgenic mice and the seven mice expressing wild-type *PS1* (using the Mann-Whitney test; a conservative test that makes no assump-

tions about sample distribution) showed that expression of wild-type *PS1* did not significantly increase the levels of A β 42(43), even though expression of wild-type *PS1* was substantially increased to levels comparable to those for mutant *PS1* (Fig. 3). However, in the 14 mice expressing mutant *PS1*, as compared with the seven mice expressing wild-type *PS1*, there was a significant increase in A β 42(43) ($P = 0.01$ for M146L, $P = 0.002$ for M146V, and $P = 0.001$ for all mutant lines combined). There was no indication in our data that the effect of the *PS1* mutations was related to expression level (Fig. 3 and Table 1). Histopathological analysis of mice at age 3–4 weeks revealed no A β deposition or other pathology (data not shown). Mice expressing sufficient amounts of the mutant APPs linked to familial Alzheimer's disease have been shown to deposit A β in senile plaques and to develop other changes like those seen in the diseased brain, but these mice do not show pathology until they are many months older^{11,12}. Thus, it will be important to determine whether mice with *PS1* mutations develop A β deposits or other abnormal phenotype as they age. It will also be of interest to determine whether breeding of these mice with mice bearing APP transgenes will lead to mice that develop Alzheimer-like pathology even if neither parental strain developed pathology on its own.

The observations reported here extend our previous findings in fibroblasts and plasma from subjects with *PS1/2* mutations in two main ways¹³. First, they show that the *PS1* mutations increase A β 42(43) not only in peripheral cells, but also in the brain, the target organ for Alzheimer's disease pathology. Thus, they validate the use of fibroblasts as a model system to study the effect of *PS1/2* mutations on β APP processing. Second, our results indicate that mutant presenilins might act in a truly dominant fashion because the normal endogenous *PS1* genes in mice expressing the mutant *PS1* transgene did not prevent the increase in A β 42(43). Thus, it seems that the pathogenic *PS1* mutations act either in a dominant-negative fashion (perhaps by forming complexes that impair overall *PS1* function) or through a gain of function that influences A β 42(43). It should be noted that we could not discriminate endogenous and transgenic *PS1* at the protein level in this study, although we could discriminate mutant and wild-type *PS1* mRNAs (Fig. 2). Endogenous *PS1* mRNA continued to be expressed even when there was marked overexpression of transgenic *PS1* mRNA (Fig. 2), making it likely that there was continued expression of endogenous *PS1* protein; however, we cannot rule out the possibility that endogenous *PS1* was down-regulated at the protein level when mutant *PS1* was overexpressed. If endogenous *PS1* is downregulated so that mutant *PS1* replaces wild-type *PS1*, then the *PS1* mutations might be acting by compromising normal *PS1* function. Additional measurements of endogenous *PS1* in transgenic animals are needed to show conclusively that the *PS1* mutation acts in a dominant fashion.

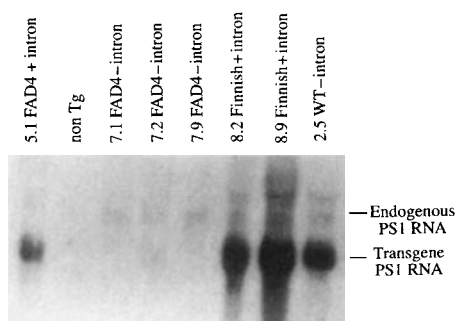


FIG. 2 Northern blot analysis of *PS1* mRNA in the brains of transgenic mice expressing wild-type or mutant *PS1*. Line numbers are given (see Table 1). The transgene mRNA band is clearly seen at ~1.6 kb as expected and the intron seems to have been spliced out correctly. A larger band at ~4 kb is also seen in transgenic animals which is thought to be a minor mRNA species arising from readthrough of the polyadenylation signal. Only the 1.6-kb transgene mRNA is translated into protein (Fig. 3). Endogenous mouse *PS1* is seen at ~3 kb. The inclusion of intron 5 generally led to greatly increased *PS1* mRNA (data not shown), as has been previously reported for other transgenes¹⁹. With the exception of line 2.5, all lines used expressed intron-containing constructs. Tg, transgenic; WT, wild type.

TABLE 1 Analysis of A β in brain tissue

Transgene	Line	Animal number	A β 40 BNT77/BA27	A β 42(43) BNT77/BC05	Increase in A β 42(43) (mutant-WT)
Non-transgenic		1	2.55	0.49	NA
		2	2.69	0.50	NA
		3	2.61	0.48	NA
		4	2.21	0.16	NA
		5	2.48	0.26	NA
		6	2.62	0.26	NA
		Mean $\pm$ s.e.m.	2.53 $\pm$ 0.07	0.36 $\pm$ 0.06	NA
PS1WT	4.2	1	2.76	0.46	NA
		1	4.55	0.74	NA
		1	2.31	0.54	NA
	4.7	2	2.70	0.54	NA
		3	2.90	0.58	NA
		4	2.98	0.56	NA
	4.8	1	2.35	0.11	NA
		Mean $\pm$ s.e.m.	2.94 $\pm$ 0.29	0.50 $\pm$ 0.07	NA
PS1 M146L	5.1	1	2.34	0.72	0.22
		2	2.52	0.69	0.19
		3	2.80	0.75	0.25
		4	2.97	0.89	0.39
	6.2	1	2.13	1.17	0.67
		Mean $\pm$ s.e.m.	2.55 $\pm$ 0.15	0.84 $\pm$ 0.09	0.34 $\pm$ 0.09
PS1 M146V	8.2	1	2.80	0.81	0.31
		2	3.21	0.82	0.32
		3	2.39	0.66	0.16
		4	2.56	0.76	0.26
		1	2.99	0.99	0.49
	8.9	2	2.71	0.90	0.40
		3	2.41	0.89	0.39
		4	2.62	0.81	0.31
		5	2.59	0.68	0.18
		Mean $\pm$ s.e.m.	2.70 $\pm$ 0.09	0.81 $\pm$ 0.04	0.31 $\pm$ 0.04

A β measurement (see text) is in pmol per g wet tissue. NA, not applicable.

Most importantly, our results indicate that presenilin-encoded Alzheimer's disease follows a pathogenic route involving A β 42(43) that may be common to all forms of the disease. Although it is possible that there are routes other than A β deposition that lead to the Alzheimer's disease phenotype, the simplest explanation, that in general it follows directly from such alterations in A β metabolism, remains unrefuted. This offers hope for a common therapeutic strategy as it would mean that most of the pathogenic process will be common to Alzheimer's disease of different aetiologies. The connection between the presenilins and β APP processing remains obscure, but both PS1 and PS2 are membrane-associated proteins, generally predicted to have seven transmembrane domains, found in the Golgi¹⁴, and which are similar to two *C. elegans* proteins, *Sel-12* (ref. 15) and *Spe-4* (ref. 8), that are involved in cell fate determination through the *lin-12/Notch* signalling pathway and spermatogenesis, respectively. The involvement of *Spe-4* in protein trafficking in the Golgi during spermatogenesis¹⁶ means that any interactions between the presenilins and β APP in this compartment might be a fruitful area of study¹⁷. □

Methods

Transgene construction. The PDGF β 2 promoter was amplified by polymerase chain reaction (PCR) from the clone psisCAT6a (ref. 18) between the *Xba*I and the *Avr*II site converting the latter to an *Xba*I site. This promoter directs neuronal expression in the CNS^{11,18}. The 1.4-kb human PS1 coding sequence (from the ATG start to the TAG stop site, lacking bases that encode the alternately spliced VRSQ motif⁶) was amplified by PCR using primers that incorporated *Not*I sites at both ends and a modified 5' Kozak sequence. A 224-bp fragment of the SV40 containing the polyadenylation signal site was amplified to incorporate *Sac*II sites using p β NASS (Clontech) as a template. The three fragments were cloned into their corresponding sites in pBluescriptII SK⁻ (Stratagene). A 2.8-kb intron between exons 4 and 5 was PCR-amplified from a human PS1 PAC clone 54-12D (ref. 5) (Genome Systems). The intron was digested with *Bcl*I and cloned

into the transgene at the *Bcl*I sites located in exons 4 and 5. The Transformer mutagenesis system (Clontech) was used to convert the wild-type sequence to M146L and M146V. Plasmid DNA was prepared using Qiagen QiaTip 100 columns and sequenced throughout. Constructs were removed from the vector by digestion with *Bss*HII, gel purification and clean up using a Wizard PCR column (Promega). DNA was eluted from the column into TE (10 mM Tris buffer, pH. 7.4, 0.1 mM EDTA) and diluted to 3–5 ng μ l⁻¹ in this buffer.

Generation and maintenance of transgenic mice. DNA was injected into fertilized eggs from a Swiss Webster (SW) (Taconic) $\times$ B6D2F₁ cross. Mice expressing 5 different constructs were generated: PS1 WT (M146) with and without intron 5, M145L with and without intron 5 and M146V with intron 5. Subsequent generations were produced by breeding founders with B6D2 or SW.

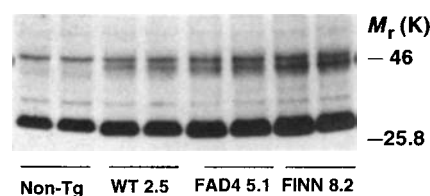


FIG. 3 Western blot analysis of PS1 in the brains of transgenic mice expressing wild-type or mutant PS1. In non-transgenic mice only the ~27K N-terminal PS1 derivative was detected. In transgenic mice expressing either wild-type or mutant PS1, there was marked overexpression of both the ~46K holoprotein and ~27K PS1 N-terminal derivative. Labelling of both the ~46K and ~27K proteins was specific because it was abolished by absorption with the cognate peptide, but labelling of the ~52K protein was not abolished, indicating that this protein is non-specifically labelled. All transgenic lines analysed for A β (Table 1) were compared on western blots (like those shown above) with equal amounts of brain lysates applied to adjacent wells. The relative amounts of ~46K holoprotein and ~27K N-terminal derivative were: WT line 4.7 < WT line 4.2 = WT line 2.5 slightly < M146L line 5.1 = WT line 4.8 = M146V line 8.2 < M146L line 6.2 = M146V line 8.9.

No difference in A β levels was observed in F₁ generations derived by crossing (SW $\times$ SW/B6D2 F₁) or (SW/B6D2 F₁ $\times$ B6D2 F₁).

Analysis of transgenic mice. To identify founders, DNA was extracted from the tails of 10 day old mice using a Genomic DNA isolation kit (Promega). DNA (10 μ g) was digested overnight with EcoRI, Southern blotted and hybridized in Quick-Hyb (Stratagene) at 68 °C for 1 hour with a ³²P-labelled PS1 complementary probe. Blots were exposed to film overnight. For northern blot analysis of mouse brains, RNA was extracted from one cerebral hemisphere of two-week-old pups using trizol reagent (Gibco/BRL). RNA was transferred to Hybond N (Amersham) as described for Southern blotting.

PCR primers and conditions. The primers used were: PDGF F, 60 °C annealing, 5' GAT CTC TAG AGG ATC CAC AG; PDGF/XbaR, 5' GAC TCT AGA GAG GCA GC; SV40 F, 5' CAT GTC GAC TAG GCG GCC GCG GGG ATC; SV40/SacII R, 5' CAT CCG CGG TCG ACT CTA GAG A; Intron 5 F, 51 °C annealing, 5' AAG ATG AGG AAG AAG ATG; Intron 5 R, 5' CCC AAC CAT AAG AAG AAC AG; PS1/NotI/Kozak F, 65 °C annealing, 5' GGC GAG CGG CCG CAC CTG CTC CGG CCA TGA CAG AGT TAC CTG CAC CA; PS1/NotI R, 5' CCG CTG CGG CCG CGG ATT CTA ACC GCA AAT ATG C. All PCR were run under standard conditions (High Fidelity Taq polymerase, Boehringer, 94 °C for 5 minutes X1, then (94 °C for 30 s, annealing temperature, for 30 s, 72 °C for 30–45 s) X30, followed by 72 °C X1 for 5 min) except for the PS1/NotI/Kozak F amplification, where the cycling extension time was increased to 5 min.

Western blot analysis. A peptide corresponding to the PS1 sequence (T2–N12) was synthesized. Two additional basic amino acids (arginine and lysine) were added to the carboxy terminus of the peptide, to increase its solubility. An additional cysteine residue was also added to conjugate the peptide to a protein carrier (ovalbumin) using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). The conjugated peptide (180 μ g) TC14. (TELPA-PLSYF-NRKC-NH₂) together with Freund's complete adjuvant, was emulsified and injected intradermally into 15–20 sites along the sides of New Zealand rabbits. Every three weeks, the rabbits were immunized using Freund's incomplete adjuvant. Ten days after the further immunization the rabbits were bled.

Mouse brain was Dounce-homogenized at a ratio of 150 mg tissue per ml of homogenization buffer (2% SDS in saline solution) containing a complete protease inhibitor cocktail (Boehringer Mannheim) and sonicated 4 times for 10 s at 50 W and 70% amplitude, using a microsonic cell disrupter (Kontes). The homogenate was spun at 35,000 g for 30 min and the supernatant was transferred to a separate tube for analysis. Protein concentration was determined using the BCA protein determination kit (Pierce). Before loading, the samples were diluted 1:1 in 2 $\times$ tricine sample buffer (Novex) containing 1% dithiothreitol. Samples were not heated before loading. The gels were electrophoresed for ~1.5 h at 125 V and transferred to Immobilon (Millipore). After blocking with 5% milk in TBS the blots were incubated with a 1:1,000 dilution of anti-PS1 antibody and detected with HRP-linked anti-rabbit secondary antibody using ECL (Amersham).

Analysis of A β in brain tissue. Tissue (150 mg) was Dounce-homogenized (6 strokes) in 1 ml of 70% formic acid. Homogenates were centrifuged at 100,000 g for 1 h. The supernatant was recovered and neutralized by a 20-fold dilution in 1 M Tris base. A sample (100 μ l) was mixed with 50 μ l of buffer EC (0.02 M sodium phosphate, 0.2 mM EDTA, 0.4 M NaCl, 0.2% BSA, 0.05% CHAPS, 0.4% Block-Ace, 0.05% sodium azide, pH 7.0) and analysed directly using the BNT77/BA27 or BNT77/BC05 sandwich ELISA systems^{20,21}. The capture antibody (BNT77) used here was raised against amino acids 11–28 of the A β peptide sequence. The values reported were calculated by comparison with a standard curve of synthetic human A β 1–40 and A β 1–42 (Bachem).

Histology. Anaesthetized mice were perfused with paraformaldehyde and horizontal brain sections were immunocytochemically stained for A β using the 4G8 antibody (Senetek, St Louis, MO) both with and without formic acid pretreatment.

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Hippocampal synaptic transmission enhanced by low concentrations of nicotine

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NICOTINE obtained from tobacco can improve learning and memory on various tasks and has been linked to arousal, attention, rapid information processing, working memory, and long-term memories that can cause craving years after someone has stopped smoking^{1,2}. One likely target for these effects is the hippocampus, a centre for learning and memory that has rich cholinergic innervation and dense nicotinic acetylcholine receptor (nAChR) expression^{3–6}. During Alzheimer's dementia there are fewer nAChRs and the cholinergic inputs to the hippocampus degenerate⁷. However, there is no evidence for fast synaptic transmission mediated by nAChRs in the hippocampus, and their role is not understood^{8,9}. Nicotine is known to act on presynaptic nAChRs within the habenula of chick to enhance glutamatergic transmission¹⁰; here we report that a similar mechanism operates in the hippocampus. Measurements of intracellular Ca²⁺ in single mossy-fibre presynaptic terminals indicate that nAChRs containing the $\alpha 7$ subunit can mediate a Ca²⁺ influx that is sufficient to induce vesicular neurotransmitter release. We propose that nicotine from tobacco influences cognition by enhancing synaptic transmission. Conversely, a decreased efficacy of transmission may account for the deficits associated with the loss of cholinergic innervation during Alzheimer's disease.

After a cigarette has been smoked, nicotine in arterial blood can reach a concentration of about 0.5 μ M, and the drug is delivered to the brain less than 10 s after absorption in the lungs¹¹. To investigate the role of hippocampal nAChRs, we applied 0.5 μ M nicotine to hippocampal cultures or pressure-puffed a low concentration onto hippocampal slices. CA3 pyramidal neurons in the rat hippocampal slice respond to nicotine with an increased rate of spontaneous miniature excitatory postsynaptic currents (mEPSCs) (Fig. 1a). Voltage-dependent Ca²⁺ channels were blocked by 200 μ M Cd²⁺, and action potentials were inhibited by 1 μ M tetrodotoxin (TTX). In those experiments, a pyramidal neuron from the CA3 region was patch-clamped in the whole-cell mode, and a pipette was positioned to apply a local pressure puff of bath solution containing 20 μ M nicotine onto the dendrites of the CA3 neuron. The actual concentration of nicotine that reached the nAChRs was much less than 20 μ M. The frequency but not the amplitude of mEPSCs increased in all four of the CA3 neurons that were tested (Fig. 1b). The time course of the frequency increase depended on the position of the puffer pipette, the depth of the neuron in the slice, and the removal of nicotine by diffusion and by wash out of the bath (1 ml min⁻¹ in a 0.6-ml chamber).

To control precisely the concentration of applied nicotine, we examined rat hippocampal neurons in tissue culture plated at a low density or on micro-islands. Nicotine (0.5 μ M) was applied to

whole-cell patch-clamped hippocampal neurons in the presence of 0.5–1.0 μM TTX. In 8 of 13 neurons, the frequency but not the amplitude of the mEPSCs increased during the nicotine application (Fig. 1c). No mEPSCs were seen when glutamate receptors were inhibited by 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 1 mM kynurenic acid in solutions containing 1 mM Mg^{2+} , indicating that the mEPSCs represent the vesicular release of glutamate.

Whole-cell currents induced by rapid applications of nicotine (0.5 mM) were measured to investigate the pharmacology of the nAChR subtype mediating the effect on glutamate release. We found that 5 nM methyllycaconitine (MLA) in all seven neurons tested, or 50 nM α -bungarotoxin (α -BGT), in all nine neurons tested, completely inhibited the rapidly desensitizing currents activated by nicotine (Fig. 2a). The MLA inhibition was reversible, but the α -BGT inhibition was not reversed even after washing for more than an hour. The kinetics and pharmacology of these currents indicate that the nAChRs contain the $\alpha 7$ subunit^{5,12–16}. Nicotine (0.5 μM) did not enhance mEPSC frequency after nAChRs were inhibited by α -BGT (Fig. 2b; $n = 6$). These results indicate that nAChRs containing the $\alpha 7$ subunit mediate the

nicotine enhancement of mEPSC frequency. In our cultures, these α -BGT-sensitive currents are by far the predominant current: only 3 of 114 neurons displayed a completely different nicotinic current with slower kinetics. Thus the α -BGT-sensitive currents mediate the nicotinic effect in our hippocampal cultures, but it remains possible that other subtypes of nAChRs could mediate these effects in other preparations where the distribution of nAChR subtypes is different. Our results do not require that the α -BGT-sensitive receptor is exclusively an $\alpha 7$ homomeric complex.

The frequency of mEPSCs did not increase when nicotine (0.5 μM) was applied to cultured neurons in a calcium-free solution (Fig. 2c). All 14 nicotine applications to cultured neurons in the absence of Ca^{2+} failed to enhance the frequency of mEPSCs. When nicotine (0.5 μM) was later applied to 10 of these same neurons in 1 mM Ca^{2+} , 7 of them showed an increase in mEPSC frequency (Fig. 2d). These results indicate that external Ca^{2+} is required for nicotine enhancement of mEPSC frequency.

Because the frequency but not the amplitude of the mEPSCs increased, the results suggest that nicotine ultimately influences presynaptic processes to increase the rate of quantal release (rather than altering the postsynaptic detection of glutamate). Previous work had indicated that nAChRs can mediate a large calcium influx and that the α -BGT-sensitive nAChR containing $\alpha 7$ has a high calcium permeability^{5,17–20}, so we investigated directly whether presynaptic calcium influx through nAChRs could underlie the effect of nicotine.

Mossy fibres from dentate granule neurons form large presynaptic terminals (3–8 μm) on CA3 neurons. The bright fluorescent signal from mossy fibre presynaptic terminals after loading with the calcium indicator fura-2 is shown in Fig. 3a. The microscope diaphragms for excitation and emission were closed down to

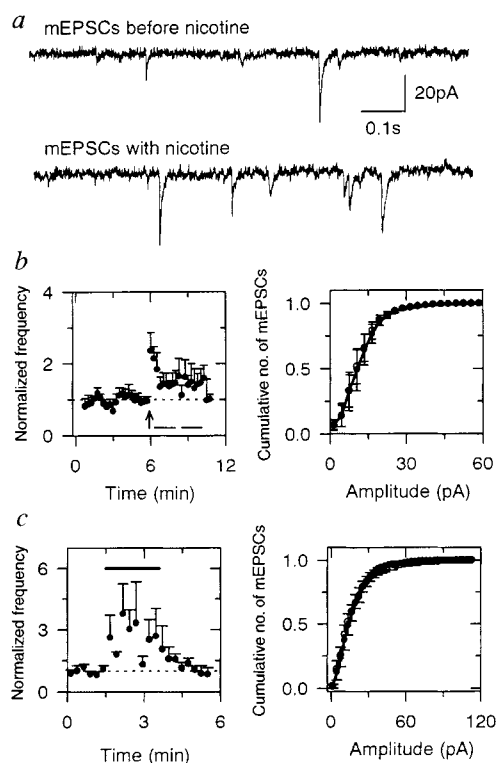


FIG. 1 Nicotine enhances the frequency but not the amplitude of mEPSCs. *a*, In rat hippocampal slice, 20 μM nicotine puffed onto the dendrites of a CA3 neuron increases the frequency of glutamatergic mEPSCs (1 μM TTX, 200 μM Cd^{2+}). Similar results were obtained in the CA1 region, but the mEPSCs were smaller and harder to analyse. *b* (left), mEPSC frequency before nicotine was normalized to 1 (dotted line). The arrow indicates the increase in mEPSC frequency shortly after the 5-s puff of 20 μM nicotine. The dashed line indicates that nicotine was present in the dendrites after the nicotine puff had stopped (6 trials from 4 neurons; $\pm$ s.e.). Right, the cumulative amplitude distribution of the mEPSCs is unchanged before nicotine (open circles) and during the enhanced mEPSC frequency with nicotine (filled circles). The total number of mEPSCs is normalized to 1 so the amplitude distributions before and after nicotine can be compared. *c* (left), With hippocampal neurons in tissue culture, glutamatergic mEPSCs are more frequent after 0.5 μM nicotine is applied (solid bar) through a large (380 μm) inflow pipe ($n = 8$, $\pm$ s.e.). Right, the cumulative amplitude distribution of mEPSCs is the same before nicotine (open circles) and during the peak of the enhanced mEPSC frequency with nicotine (filled circles).

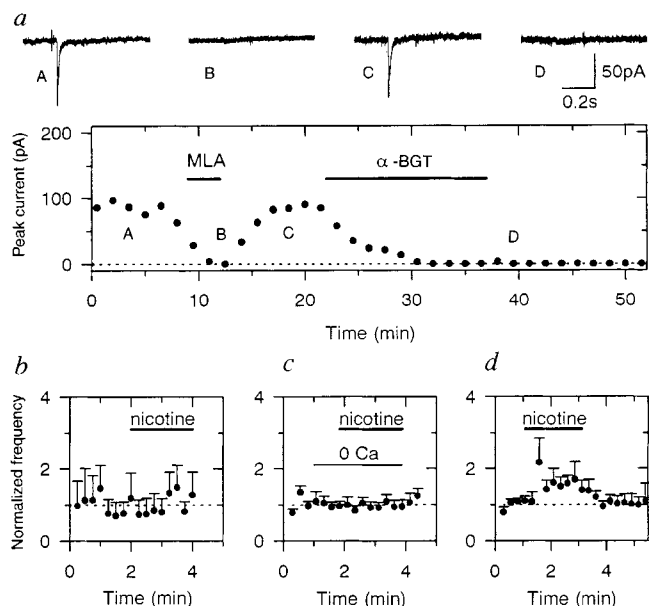


FIG. 2 Nicotine-mediated enhancement of mEPSC requires external Ca^{2+} and is inhibited by α -BGT. *a*, Four nicotine-induced (0.5 mM) currents are shown (A–D) (average of three nicotine applications). The time course for inhibition of these currents by 5 nM MLA (short bar) or 50 nM α -BGT (long bar) is shown underneath. *b*, Neurons were pretreated with 50 nM α -BGT, which inhibits α -BGT-sensitive nAChRs. Nicotine (0.5 μM , solid bar) does not increase the frequency of mEPSCs after α -BGT treatment ($n = 6$, $\pm$ s.e.). *c*, In a Ca^{2+} -free external solution (solid line), nicotine (0.5 μM , solid bar) did not increase the frequency of mEPSCs ($n = 14$, $\pm$ s.e.; 0.5 μM TTX, 200 μM Cd^{2+}). *d*, With the same neurons studied in zero- Ca^{2+} , when 1 mM Ca^{2+} was included in the external solution, nicotine (0.5 μM , solid bar) increased the frequency of mEPSCs ($n = 7$, $\pm$ s.e.; 0.5 μM TTX, 200 μM Cd^{2+}).

include in the centre of the field of view the brightest mossy terminal, which was 4 μm in diameter and was near the surface of the slice. When bath solution containing 20 μM nicotine was puffed locally onto that terminal, the fluorescence in response to excitation at a wavelength of 380 nm decreased, indicating that intracellular Ca^{2+} increased (Fig. 3b). In separate experiments, puffs of solution without nicotine ($n = 7$) did not alter the fluorescent signal.

To verify that the increase in calcium was exclusively presynaptic, postsynaptic ionotropic glutamate receptors were shown to have no effect on the fura-2 signal. Mossy terminals were loaded with fura-2, and a presynaptic Ca^{2+} influx was induced through voltage-dependent Ca^{2+} channels by stimulating the mossy fibre pathway with an electrode placed in the dentate region (Fig. 3c). Before and after blocking postsynaptic glutamate receptors with 100 μM 2-amino-5-phosphonopentanoic acid (AP5) and 20 μM CNQX, the Ca^{2+} influx detected by fura-2 was identical, indicating that a postsynaptic Ca^{2+} influx mediated by glutamate receptors was not contaminating the presynaptic measurement. Stimulation did not increase presynaptic Ca^{2+} after inhibiting Na^+ channels and voltage-dependent Ca^{2+} channels (Fig. 3c, +TTX, Cd). Inhibition of the ionotropic glutamate receptors also had no effect on the nicotine-induced influx of Ca^{2+} (Fig. 3d). As found in tissue culture, α -BGT (100 nM) inhibited the effect ($n = 3$ of 3), indicating that $\alpha 7$ -containing nAChRs predominantly mediate the rise in $[\text{Ca}^{2+}]_i$.

The Ca^{2+} signal through nAChRs is significant when compared to the Ca^{2+} influx through presynaptic voltage-dependent Ca^{2+} channels (Fig. 3e). In this experiment, glutamate receptors were inhibited while the mossy fibre pathway was stimulated by an electrode placed in the dentate region. Stimulation of the voltage-dependent Ca^{2+} channels continued while 20 μM nicotine was puffed onto the presynaptic terminal. The fluorescent signal ($\Delta F/F$) induced by stimulation of these Ca^{2+} channels was $13.7 \pm 3.0\%$ ($n = 4$), where F is the intensity of the fluorescence; $\Delta F/F$ induced by the nicotine puff was $13.4 \pm 5.4\%$ ($n = 9$ of 9). Thus the average increase in $[\text{Ca}^{2+}]_i$ caused by nicotine was similar to that

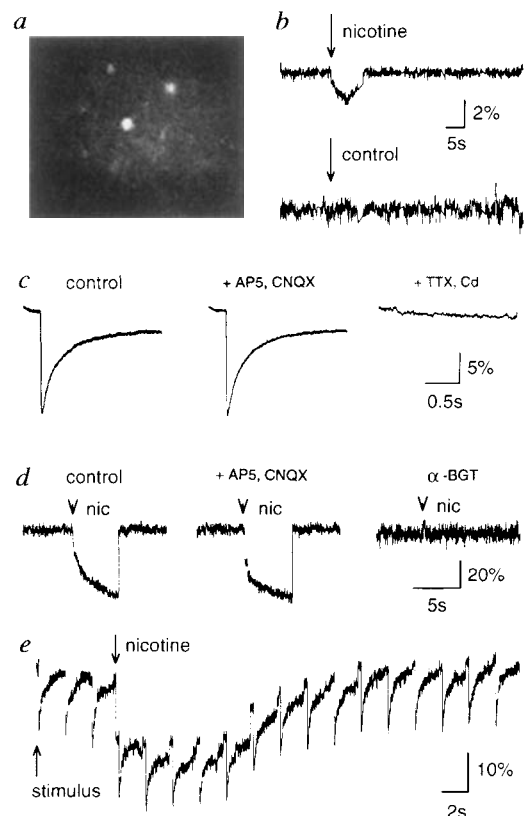
produced by an action potential invading the presynaptic terminal.

It is likely that voltage-dependent Ca^{2+} channels and nAChRs are distributed differently and that the Ca^{2+} concentration is higher in those local presynaptic domains that are responsible for transmitter release²¹. It is clear, however, that there is a large Ca^{2+} influx through nAChRs averaged over the whole presynaptic mossy fibre terminal that could contribute to the enhanced glutamate release described earlier (Figs 1 and 2). The results further suggest that presynaptic activation of nAChRs by cholinergic afferents might be able to induce glutamate release without requiring an action potential along the axon leading to that glutamatergic terminal.

Normally, when an action potential reaches a presynaptic terminal, voltage-dependent Ca^{2+} channels mediate an increase in $[\text{Ca}^{2+}]_i$, but that does not ensure that neurotransmitter will be released; rather, there is some probability of release. If presynaptic nAChRs open just before the arrival of an action potential, presynaptic $[\text{Ca}^{2+}]_i$ is elevated, increasing the probability of neurotransmitter release, a mechanism similar to that for paired-pulse facilitation. During the first of a pair of stimulated action potentials, not all presynaptic terminals release transmitter, but intra-terminal Ca^{2+} remains elevated for ~ 200 ms (refs 22, 23). If the second action potential arrives while Ca^{2+} is still elevated, there is enhanced release (facilitation). Similarly, if presynaptic nAChRs are activated just before the arrival of an action potential, presynaptic Ca^{2+} will be greatly increased. Because transmitter release usually depends on the fourth power of intra-terminal Ca^{2+} concentration, nAChR activity could enhance release and ensure the success of synaptic transmission. Thus, properly timed nAChR activity could ensure that important events emerge out of the noise of synaptic probabilities. It is this fidelity that may be diminished by the loss of nicotinic function in the hippocampus during Alzheimer's disease.

These results are consistent with previous findings that exogenous nicotine acts on presynaptic nAChRs to enhance glutamatergic synaptic transmission in chick neurons from the habenula and

FIG. 3 Nicotine elevates Ca^{2+} in mossy-fibre presynaptic terminals. *a*, High-contrast, low-brightness image of a hippocampal slice showing several mossy fibre presynaptic terminals loaded with fura-2 (bright white spots). *b*, The fura-2 fluorescence ($\Delta F/F$) excited at a wavelength of 380 nm, where the arrow indicates a 1-s puff of 20 μM nicotine (1 μM TTX, 200 μM Cd²⁺, 100 μM AP5, 20 μM CNQX). The control shows that a puff of solution without nicotine has no effect. The traces were constructed from six records each of 10 s, separated by a gap of 10 s (gaps not shown). *c*, Presynaptic Ca^{2+} influx was induced through voltage-dependent Ca^{2+} channels by stimulating the mossy fibres. The Ca^{2+} influx indicated by the sharp decrease in $\Delta F/F$ was the same in control bath solution and after adding 100 μM AP5 and 20 μM CNQX to block postsynaptic glutamate receptors. Adding 1 μM TTX and 500 μM Cd²⁺ inhibited Na^+ channels and voltage-dependent Ca^{2+} channels and blocked the stimulus-induced fluorescence change. Each $\Delta F/F$ trace is the average of >11 individual traces. *d*, The nicotine-induced (5-s puff, arrowhead) $\Delta F/F$ is the same before (control) and after inhibiting glutamate receptors with 100 μM AP5 and 20 μM CNQX. Preincubation of the slice with 100 nM α -BGT inhibited the nicotine-induced $\Delta F/F$. The traces were constructed from three records each of 5 s, separated by a gap of 10 s (gaps not shown). *e*, A record of $\Delta F/F$ (100 μM AP5 and 20 μM CNQX) with the mossy fibres stimulated at 0.1 Hz (one stimulus indicated by the upward arrow). A 2-s puff of 20 μM nicotine (just before downward arrow) caused the $\Delta F/F$ to decrease (a fall in the baseline), indicating that the increase in presynaptic Ca^{2+} was about the same as that induced by stimulation of the voltage-dependent Ca^{2+} channels. The trace was constructed from 18 records each of 2 s, separated by a gap of 10 s (gaps not shown).



from the visceral motor nucleus of Terni¹⁰. Nicotine also has been found to enhance the release of excitatory, inhibitory and modulatory neurotransmitters from synaptosomes²⁴. Finally, these results indicate that a smoker's level of nicotine can act on presynaptic nAChRs in the hippocampus to alter synaptic transmission. There are a myriad of psychopharmacological effects caused by nicotine²⁵. The effects on working memory may be partly explained by the ability of nicotine to enhance or induce neurotransmitter release in the hippocampus. □

Methods

Hippocampal slices were prepared from Sprague-Dawley rats (aged 20–63 days) using standard techniques²⁶. Slices 200–400 µm thick were cut in cold solution (in mM: 124 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 dextrose) bubbled with 95% O₂, 5% CO₂. The bath solution contained (in mM) 149 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 26 NaHCO₃, 10 dextrose, bubbled with 95% O₂ and 5% CO₂, and, unless stated otherwise, 1 µM TTX and 200 µM CdCl₂ were present. The solution in the puffer pipette was the same except the buffer was 10 mM HEPES, pH 7.3, and 20 µM nicotine was added. Neurons were visualized for patch-clamp recordings under infrared light using Nomarski optics²⁷. The mEPSC data were filtered at 2 kHz and were collected onto VHS tape (via an Instrutech VC-10). The holding potential was –60 mV. The data were then sampled and analysed from tape as described below.

Tissue-cultured neurons were obtained from whole hippocampi of postnatal (<3 days) rats²⁸. Nicotine-induced currents were larger and more commonly expressed after 10 days in culture (refs 12–15), so we studied the neurons on days 10–30. The external bath and control solution contained (in mM) 150 NaCl, 2.5 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, 0–200 µM CdCl₂, 20–100 µM picrotoxin, 0.5–1 µM TTX, pH 7.3; 10 µM CNQX was added for the pharmacology experiments (Fig. 2a). The pipette solution for whole-cell patch-clamp recordings contained (in mM) 150 CsCH₃SO₃, 5 NaCl, 0.2 EGTA, 2–5 Mg-ATP, 0–2 Na₂ATP, 0–0.3 Na₃GTP, 10 HEPES, pH 7.3. Standard patch-clamp techniques were used²⁸. In some cases, perforated patches were used²⁹. The holding potential was –50 or –70 mV. Currents were amplified and filtered (1–2 kHz) using an Axopatch 1B or 1D voltage clamp with a 4-pole Bessel filter and were digitally sampled to exceed the Nyquist criterion. All experiments were conducted at room temperature (about 23 °C). All programs were written in Axobasic (Axon), and a program from the laboratory of C. F. Zorumski was adapted for the off-line analysis of mEPSCs. For the plots of mEPSC frequency, bins of 15 s were used, and for the amplitude distributions bins of 3 pA were used. Animal care was in accordance with institutional guidelines.

The pharmacology experiments (Fig. 2) relied on our finding that, with hippocampal neurons in culture >60% of the time, 0.5 µM nicotine enhanced mEPSC frequency. Therefore, if MLA and α-BGT did not inhibit the effect of nicotine, >60% of the nicotine applications should increase mEPSC frequency. Neuronal cultures were pretreated for >8 min with 50 nM α-BGT, which should inhibit all α-BGT-sensitive nAChRs. On average, after α-BGT treatment, nicotine did not increase mEPSC frequency (Fig. 2b), but in one of six α-BGT experiments in culture, mEPSC frequency did increase either coincidentally or because some synapses possess α-BGT-insensitive nAChRs that mediate the effect.

The loading of mossy fibre terminals in the hippocampal slices with cell-permeant fura-2 acetoxymethyl ester (fura-2AM) took advantage of two findings during preliminary experiments. First, CA3 neurons in the hippocampal slice do not load well with fura-2AM; second, presynaptic terminals tended to load well with other fluorophores. We therefore began loading slices for 10–15 min with fura-2AM (50 µg added to 40 µl of 20% Pluronic in DMSO to make 1.25 mM stock). The fura-2AM stock was added to the bath solution of the slice at a final concentration of 8.3 µM. With a silicon-intensifier target camera at high contrast and low brightness, 380-nm excitation caused large mossy fibre terminals to stand out brightly from the poor background loading of the slice. The emitted light passed through a 510-nm long-pass filter and was focused onto a single photodiode (Hamamatsu). The fluorescence change (ΔF) induced by stimulation or nicotine was divided by the total fluorescence (F) to normalize for changes in the baseline that could produce artefacts arising from changes in cellular morphology, fura-2 concentration, or bleaching³⁰. Presynaptic terminals close to the surface of the slice were studied to improve the optics and optimize the pressure-puff applications of nicotine.

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Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75

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MEMBERS of the nerve growth factor (NGF) family promote the survival of neurons during development¹. NGF specifically activates the receptor trkA, initiating a signal transduction cascade which ultimately blocks cell death. Here we show that NGF can have the opposite effect, inducing the death of mature oligodendrocytes cultured from postnatal rat cerebral cortex. This effect was highly specific, because NGF had no effect on oligodendrocyte precursors and astrocytes. Other neurotrophins such as brain-derived neurotrophin factor (BDNF) and neurotrophin-3 (NT-3) did not induce cell death. NGF binding to mature oligodendrocytes expressing the p75 neurotrophin receptor, but not trkA, resulted in a sustained increase of intracellular ceramide and c-Jun amino-terminal kinase (JNK) activity, which are thought to participate in a signal transduction pathway leading to cell death. Taken together, these results indicate that NGF has the ability to promote cell death in specific cell types through a ligand-dependent signalling mechanism involving the p75 neurotrophin receptor.

Oligodendrocytes are responsible for myelination in the central nervous system. Bipolar oligodendrocyte progenitor cells from neonatal rat cortex were separated from mixed glial cultures by differential shaking² and allowed to differentiate in the presence of serum-free medium (differentiation medium)³. After seven days in culture, mature oligodendrocytes were identified by their elaborate processes and strong immunoreactivity for galactocerebroside and myelin basic protein⁴.

After incubation with NGF for four hours, cell death was observed in long-term cultures of mature oligodendrocytes, as assessed by red fluorescence due to ethidium bromide binding to

DNA in condensed cell nuclei (Fig. 1b). Fluorescence-based TUNEL measurements also detected numerous apoptotic nuclei after NGF treatment. Oligodendrocyte precursor cells and astrocytes derived from the same mixed cultures were unaffected by NGF treatment (Fig. 1d,f).

The effect of NGF on mature oligodendrocytes required high concentrations (100 ng ml^{-1}) and showed rapid kinetics (Fig. 2). After four hours, 40% of the cells were dead. Similar kinetics have been observed in CD95 (Fas)-transfected L929 and WR19L cells⁵ and in oligodendrocyte cells treated with ceramide analogues⁴. Although the percentage of dead cells observed after overnight treatment was not higher than that observed at four hours, this result is likely to be an underestimate, because of detachment of dead cells. The effect of NGF on oligodendrocyte viability was specific, as treatment with other neurotrophins, such as BDNF or NT-3 (100 ng ml^{-1}), had no detectable effect (Fig. 2).

NGF can bind independently to two different receptors, the trkA tyrosine kinase receptor and the p75 neurotrophin receptor. The trkA receptor is directly responsible for neuronal cell survival, whereas the p75 receptor can modulate trkA activities when coexpressed at high levels⁶. Previous results with pig oligodendrocytes indicated a proliferative effect of NGF⁷, which may be due to trkA expression. To investigate whether trkA was expressed by rat oligodendrocytes, reverse transcription-polymerase chain reaction (RT-PCR) was carried out. Whereas dorsal root ganglia RNA expressed trkA, RNA isolated from oligodendrocytes, cultured as described in Methods, was found to be negative (Fig. 3), consistent with previous measurements⁸. In contrast, mature oligodendrocytes cultured for seven days in DM medium were found to express p75, as detected by immunocytochemistry (Fig. 3a) western blot analysis (Fig. 3d, lane 3) and RT-PCR analysis (Fig. 3e). The p75 receptor could not be detected in cultured oligodendrocyte precursor cells (Fig. 3b and d, lane 2) or astrocytes (Fig. 3c and d, lane 1).

The expression of p75, and not trkA, in differentiated oligodendrocytes indicated that this neurotrophin receptor may generate a cell-death signal. Owing to the similarity in the overall receptor structure of p75 with related family members, such as the p55 tumour necrosis factor (TNF) receptor and the CD95/Fas antigen⁹, it has been assumed that p75 may have a similar death-inducing function. The involvement of p75 in the death of mature oligodendrocytes by NGF was further supported by experiments with blocking antibodies. Incubation of mature oligodendrocytes with 1:100 dilution of the REX antibody directed against the extracellular domain of the p75 receptor¹⁰ in the presence of 100 ng ml^{-1} NGF negated the cell-death effects, whereas treatment with antibodies against trkA (RTA)¹¹ did not affect cell viability or interfere with NGF-mediated apoptosis of oligodendrocytes (Fig. 2).

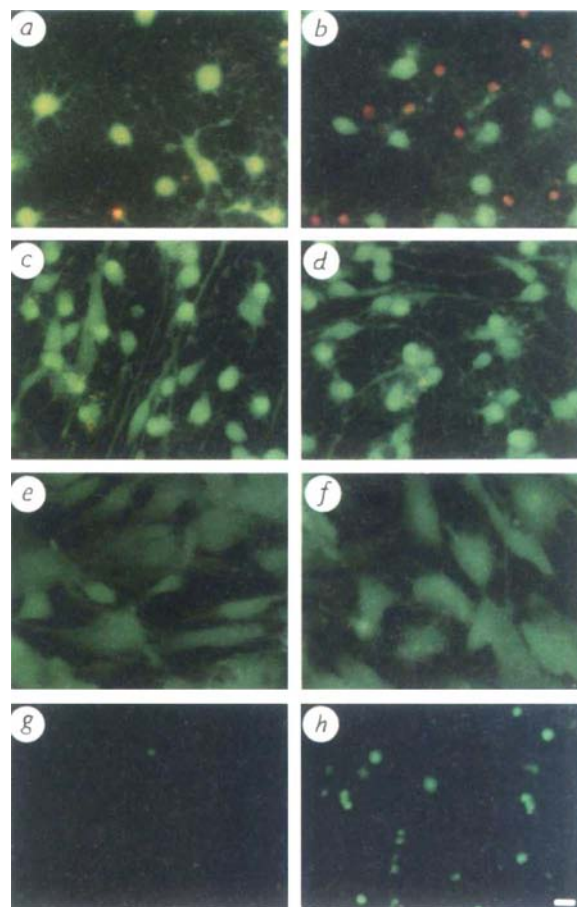


FIG. 1 NGF induces apoptotic death of mature oligodendrocytes and not oligodendrocyte precursors or astrocytes. Live/dead (a–f) and TUNEL (g, h) staining of rat cortical primary cells treated with 100 ng ml^{-1} NGF (b, d, f, h) or left untreated (a, c, e, g). In the live/dead staining (a–f), viable cells showed bright green fluorescence, owing to intracellular esterase activity on calcein acetoxymethyl ester (AM), whereas dead cells were identified by the red fluorescence, owing to incorporation of ethidium D1. In the TUNEL staining (g, h), untreated cells (g) displayed few fluorescent nuclei, whereas NGF-treated oligodendrocytes (h) were labelled. Scale bar, $25 \mu\text{m}$.

FIG. 2 Specificity of NGF upon the survival of differentiated oligodendrocytes. Quantitation of the effects of neurotrophins on oligodendrocyte survival. Left panel, Dose-response of 4-h treatment with recombinant NGF. Centre panel, Effect of 4-h treatment with 100 ng ml^{-1} of BDNF, NT-3, or $10 \mu\text{M}$ ceramide (CER). There was no difference in the number of dead cells between samples treated with 5 ng ml^{-1} NGF (14% dead cells ± 1 , $n = 4$), 20 ng ml^{-1} NGF (16.8% dead cells ± 10 , $n = 11$) and controls (13% ± 7 , $n = 11$). In contrast, at 100 ng ml^{-1} NGF induced death in 39.3% dead cells ± 11 ($n = 13$) of the mature oligodendrocytes. Right panel, Effects of antibodies against the extracellular domain of p75 (ref. 10) (REX + NGF) or against the extracellular domain of rat trkA¹¹ (RTA + NGF).

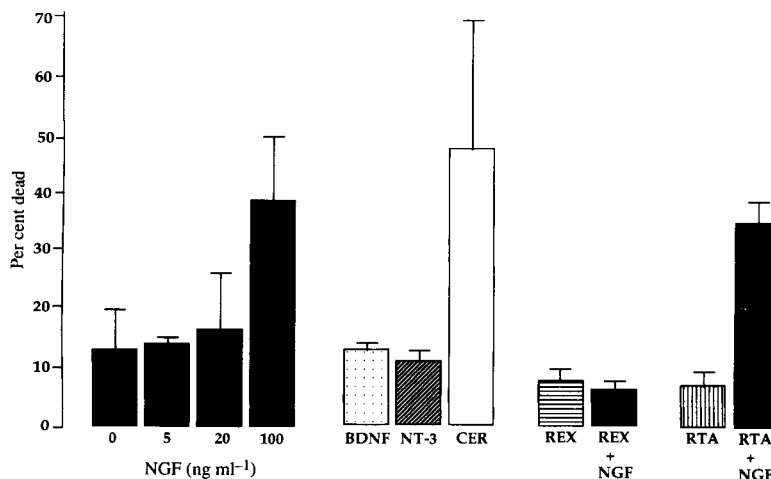
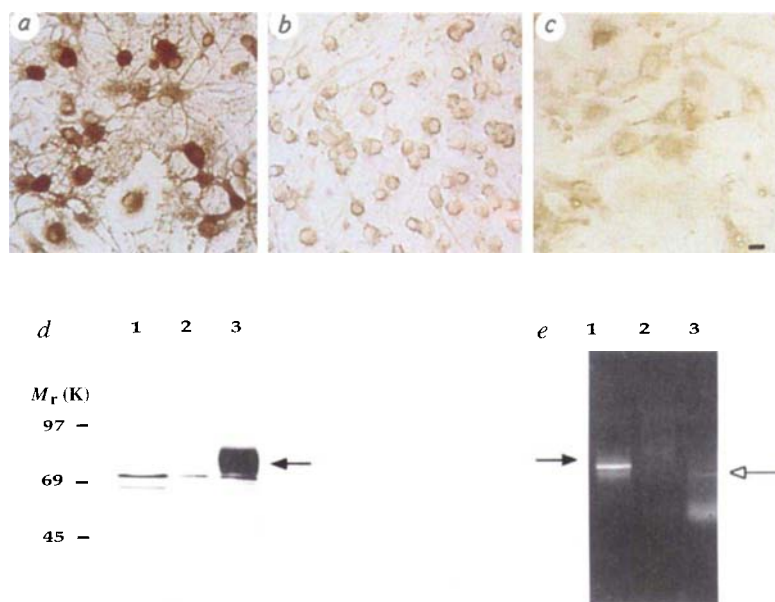


FIG. 3 Mature oligodendrocytes express p75, and not *trkA*. Immunoreactivity for p75 receptor expression was assessed for **a**, rat cortical cultures of mature oligodendrocytes; **b**, oligodendrocyte precursors; **c**, primary cortical astrocytes. Scale bar, 25 μm . **d**, Western blot analysis of whole-cell lysates isolated from cultured cells: lane 1, astrocytes; lane 2, oligodendrocyte precursors; lane 3, differentiated oligodendrocytes. The p75 protein (arrow) could be observed in mature oligodendrocytes, but not in astrocytes or precursor cells. **e**, RT-PCR analysis of *trkA* and p75 messenger RNA expression. The solid arrow indicates the presence of the p75 NGF receptor transcript in primary oligodendrocytes (lane 1). In contrast, *trkA* transcripts could not be amplified from the same cDNA (lane 2). As a positive control for the detection of *trkA* receptors, RT-PCR was performed on RNA isolated from dorsal root ganglia neurons (lane 3, open arrow).



Binding of NGF to p75 receptors has been shown to elevate intracellular ceramide levels through increased sphingomyelin hydrolysis¹². As increased ceramide has been associated with apoptosis^{13–15}, levels of intracellular ceramide were measured in differentiated oligodendrocytes after NGF treatment. In contrast to previous kinetic measurements in glioma cells¹², NGF led to an elevation of ceramide levels that was sustained for a period of several hours (Fig. 4). Interestingly, other neurotrophins such as BDNF (data not shown) and NT-3, which also bind to p75 (ref. 16), did not induce long-term ceramide production in mature oligodendrocytes. These data suggest that the high sustained ceramide levels elicited by the p75 receptor may contribute to NGF-dependent death.

To investigate the signalling components responsible for cell death of primary cortical oligodendrocytes, the activity of JNK was assessed. The JNK enzymatic activity, emblematic of the stress-activated protein kinase family, has been implicated in cell-death progression in PC12 cells¹⁷. In addition, ceramide has been found to be an activator of JNK^{15,18}. To assess JNK activity, an immune-complex kinase assay was applied to cultured oligodendrocytes. Significantly, incubation of mature oligodendrocytes with 100 ng ml⁻¹ NGF for three hours led to a twofold induction of JNK activation (Fig. 5). The activation of JNK activity in oligodendrocytes by NGF was similar to the effects of ceramide or osmotic shock. Treatment with 5–10 μM C2-ceramide induced JNK activation. These concentrations also induced oligodendrocyte cell death⁴. As a control for the specificity of C2-ceramide action, application of the analogue dihydro-C2-ceramide (dHC2; ref. 19), did not enhance JNK activation of oligodendrocytes (Fig. 5). This was consistent with the inability of dHC2 to induce death⁴. Ceramide treatment of astrocyte cultures, which are refractory to NGF-induced cell death, did not result in any change in JNK activity (data not shown). An increase in JNK activity was also not observed in p75-expressing NIH3T3 fibroblasts (Fig. 5), cells whose viability was not affected by NGF.

It should be noted that the activation of JNK by NGF binding to the p75 receptor, and the sustained level of ceramide, has so far been observed only in mature oligodendrocytes. Strikingly, the p75-3T3 cells respond to all neurotrophins with a transient increase in ceramide levels^{12,20}, but do not undergo cell death after neurotrophin treatment. These data indicate that there is a dichotomy of effects elicited by p75 which is extremely cell specific. Differences between a transient elevation and a prolonged increase in ceramide levels may result in separate signalling

cascades and different biological responses, depending on the cell type and differentiative stage. It should be stressed that the increases in ceramide and JNK activity remain correlative with the cell death observed here. It is clear, however, that the apoptotic action of NGF is highly restricted to specific cell types. Indeed, there is little evidence that Schwann and melanoma cells expressing high levels of p75 undergo neurotrophin-dependent cell death.

A pro-apoptotic effect of p75, in the absence of binding of NGF, has been proposed in studies using immortalized neuronal cell lines²¹ and in postnatal sensory neurons²². In contrast to a ligand-independent mechanism, the experimental system reported here demonstrates that NGF binding to p75 directly produces a long-term ceramide increase and activation of JNK that is correlated with oligodendrocyte cell death in the absence of *trkA* expression. Although p75 can influence *trkA* binding²³ and signalling^{24,25}, p75 can also display independent signalling properties through ceramide production^{12,20} and increased NF κ B activity²⁶. Signalling by p75 seems to be limited to NGF, as BDNF and NT-3 are ineffective. These results are paradoxical, as both BDNF and NT-3 can bind to p75 (ref. 16). Though all three neurotrophins are ligands for p75, there must be a mechanism to dictate responsiveness only with NGF.

The relevance of p75-mediated cell death of oligodendrocytes

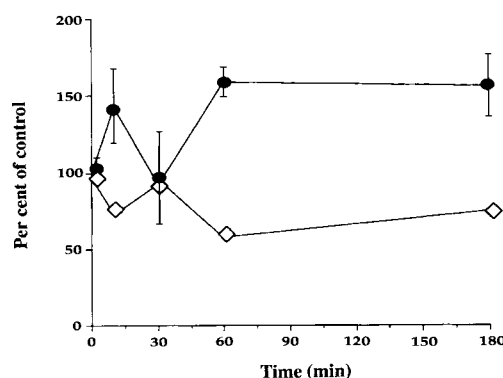


FIG. 4 NGF elicits a prolonged increase in ceramide levels. Time course in minutes of ceramide production in cultures treated with either 100 ng ml⁻¹ NGF (filled circles; $n = 6$) or NT-3 (open diamonds; $n = 2$).

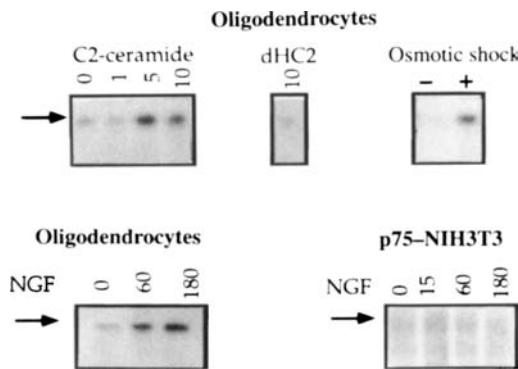


FIG. 5 NGF activates JNK activity in mature oligodendrocytes and not in 3T3 cells overexpressing p75. Activation of JNK activity by 3 h of treatment with C2-ceramide (1–10 μ M) or 100 ng ml⁻¹ NGF (0–180 min). An inactive ceramide, 10 μ M dihydro-C2-ceramide (dHC2), did not activate JNK. Osmotic stress was used as positive control for JNK activation. NGF treatment (100 ng ml⁻¹) for 0–180 min induced JNK activation in oligodendrocytes, but not in p75-expressing NIH3T3 fibroblasts. Arrow indicates GST-c-Jun (1–79).

remains unclear. During development, considerable oligodendrocyte cell death occurs in the optic nerve²⁷. Whereas the majority of oligodendrocytes do not express p75 *in vivo* in physiological conditions, these glial cells are highly susceptible to injury and inflammation. The sensitivity of oligodendrocytes is underscored by the effects of ceramide and other cytokines, such as TNF- α , which cause cytotoxicity at concentrations that do not affect other cell types, such as astrocytes or neuronal populations^{4,28}. During traumatic lesion, it is conceivable that oligodendroglial cells become more reactive to growth factors and cytokines that are released at the site of injury. Experimental support for this idea comes from the detection of p75 receptors in oligodendrocytes under pathological or neurodegenerative conditions, after kainate lesion, for example²⁹. Additional investigation into the state of reactive glial cells during multiple sclerosis or spinal cord injury may also indicate an *in vivo* role for neurotrophin-dependent cell killing.

Note added in proof: Evidence for ligand-dependent-cell death by p75 has been reported (Frade, J. M. *et al. Nature* **383**, 166–168 (1996)). □

Methods

Cell culture. Primary cortical cultures (PO) of oligodendrocytes and astrocytes were obtained from differential shaking of mixed glial cultures kept in M15 media (MEM containing 15% fetal calf serum) for 7 d. Immediately after shaking, precursor cells were plated on poly-lysine-coated dishes in M15 medium. After 6–18 h, precursor cells were either kept proliferating in MM medium (DMEM containing 30% B104 conditioned medium + N1 supplement: 5 μ g ml⁻¹ transferrin, 16 μ g ml⁻¹ putrescine, 60 ng ml⁻¹ progesterone, 50 ng ml⁻¹ selenium, 5 μ g ml⁻¹ insulin) or immediately differentiated to oligodendrocytes in DM medium, consisting of BME:F12 (1:1) supplemented with 50 μ g ml⁻¹ transferrin, 5 μ g ml⁻¹ putrescine, 3 ng ml⁻¹ progesterone, 2.6 ng ml⁻¹ selenium, 12.5 μ g ml⁻¹ insulin, 0.4 μ g ml⁻¹ thyroxine, 0.3% glucose and 6 mM glutamine. Astrocytes (GFAP⁺) were maintained in M15 medium. Oligodendrocytes (O1⁺, MBP⁺) were used after 7 d in differentiation medium (DM).

Cell-death measurements. Before treatment, cells were washed with serum-free medium and then incubated with 100 ng ml⁻¹ of recombinant NGF or BDNF or NT-3 or with different concentrations of C2-ceramide, and then analysed with live/dead stain (Molecular Probes). After live/dead staining, red and green fluorescent cells were counted. Antibodies against p75 (REX) or trkA (RTA) were added at a 1:100 dilution at the same time as NGF. For detection of apoptotic nuclei, cells were fixed in 4% paraformaldehyde, permeabilized in buffer containing 0.1% sodium citrate, 0.1% Triton X-100 and labelled with the TUNEL reaction mixture (Boehringer).

Immunocytochemistry and Immunoblot. Cultures were fixed in 4% paraformaldehyde and stained with an antiserum against the extracellular domain of the p75 receptor, 9651 (ref. 30), at a 1:1000 dilution. Positively immunoreactive cells were visualized using the ABC Elite kit (Vector Laboratories). Whole-cell lysates (200 μ g protein per lane) were separated with 10% acrylamide SDS-PAGE, transferred onto PVDF membranes and blotted with an immunopurified antiserum (9992) against the intracellular portion of p75 receptor (1:2,000). Positive proteins were visualized by chemiluminescence.

PCR analysis. Total RNA (5 μ g) was isolated from differentiated oligodendrocytes (lanes 1 and 2) or neonatal DRG (lane 3) and reverse transcribed into complementary DNA using specific primers for either rat trkA or p75. The cDNA was then amplified using 40 cycles with the following conditions: 94 °C for 50 s, annealing at 55 °C for 60 s, extension at 72 °C for 60 s. Primers specific for p75 (sense GTCGTGGGCTTGTGG, corresponding to nucleotides 903–918; antisense: GTGAGTTACACTGGGG, corresponding to nucleotides 1381–1397) and for trkA (sense GTTGATGCTGGCTTGTG, corresponding to nucleotides 136–152) and antisense: GGAGAGATTCAGGTGACTGA, corresponding to nucleotides 411–430).

Ceramide determination. Cultures were treated with different neurotrophins (100 ng ml⁻¹) for different time points and cells were scraped into methanol. Lipids were extracted and ceramide levels determined using the diacylglycerol kinase assay¹².

C-Jun kinase assay. Cultured oligodendrocytes were treated with 100 ng ml⁻¹ NGF or with C2-ceramide or dihydro-C2 in serum-free medium at the indicated concentration and time (Fig. 5). Osmotic stress consisted of exposure to 0.5 M NaCl for 30 min. Cells were collected in Triton lysis buffer and immunoprecipitated using anti-JNK antibodies (Santa Cruz). Phosphorylation of GST-c-Jun (1–79) protein was carried out as in ref. 18 and quantitated by phosphorimager analysis (Molecular Dynamics).

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Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product

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RECOGNITION of virus-infected cells by CD8⁺ cytotoxic T lymphocytes requires that the viral proteins be processed into peptides, the derived peptides transported into the endoplasmic reticulum and inserted into the binding groove of a major histocompatibility complex class I molecule, and the antigenic complex exported to the cell surface¹. However, viral pathogens can disrupt this process and interfere with immune recognition¹⁻⁴. These mechanisms may be vital to large viruses such as human cytomegalovirus (CMV), which causes persistent infection despite producing over 200 potentially antigenic proteins during the sequential immediate-early, early and late phases of viral gene expression^{5,6}. Products of CMV early-phase gene expression can globally block class I presentation⁷⁻¹⁰ and prevent recognition of infected cells by cytotoxic T lymphocytes, but an essential viral transcription factor, the 72K principal immediate-early protein, is abundantly expressed before this blockade. However, only a few host CD8⁺ cytotoxic T lymphocytes specific for immediate-early protein are present in seropositive individuals, and these lyse CMV-infected cells poorly¹¹. Here we demonstrate selective abrogation of immediate-early peptide presentation by a CMV matrix protein with associated kinase activity and suggest that modification of a viral protein can result in limiting access to the processing machinery and evasion of cytotoxic-T-cell recognition.

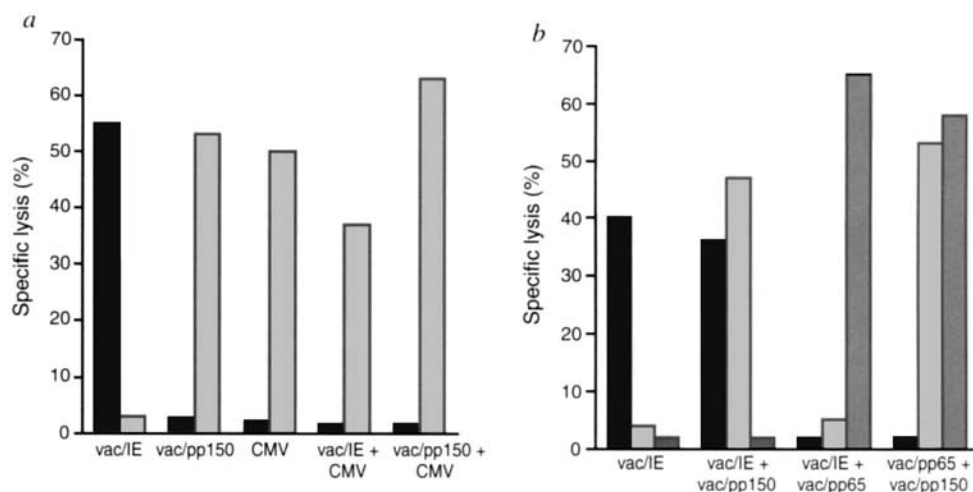
Immediate-early-peptide(IE)cytotoxic lymphocytes (CTLs) efficiently lyse autologous cells infected with recombinant vaccinia virus encoding this protein (vac/IE) but not cells infected with CMV, although both targets express the protein¹¹; this could be the result of an inhibitory effect by other CMV proteins or of help

from vaccinia proteins in the recombinant virus. We therefore infected autologous fibroblast targets with either a single virus or with combinations of CMV, vac/IE, or a control virus vac/pp150 (encoding the CMV pp150 matrix protein)¹². IE-specific CTLs lysed targets infected with vac/IE alone but failed to lyse CMV-infected targets or targets co-infected with CMV and vac/IE, despite comparable levels of IE expression (Figs 1a, 2a). By contrast, CTLs specific for pp150 lysed with equal efficiency targets infected with vac/pp150 or CMV alone, or targets co-infected with vac/pp150 and CMV or vac/IE and CMV (Fig. 1a). Thus, a CMV protein appears selectively to interfere with IE presentation, but not with presentation of other viral proteins.

Several CMV virion matrix proteins are introduced into the cell during viral entry and are detectable before IE gene expression starts¹²⁻¹⁴. CMV-infected cells treated sequentially with metabolic inhibitors of translation and transcription to permit expression of only IE genes are efficiently lysed by CTLs specific for introduced matrix proteins but not those specific for IE, suggesting that virion proteins are candidates for inhibiting presentation of IE (data not shown)^{15,16}. As the most abundant CMV matrix proteins are pp150 and pp65 (ref. 17), we co-infected fibroblasts with combinations of vac/IE, vac/pp150, vac/pp65 and wild-type vaccinia to assess blockade of IE presentation^{11,12}. We found that IE-specific CTLs lysed cells co-infected with vac/IE and either vaccinia or vac/pp150, but not cells co-infected with vac/IE and vac/pp65 (Fig. 1b). By contrast, CTLs specific for pp65 lysed targets co-infected with vac/IE and vac/pp65, and pp65 expression did not interfere with lysis of pp150-expressing targets by pp150-specific CTLs (Fig. 1b). This selective inhibition by pp65 was not limited to a single IE epitope as other IE-specific CTL clones, which were restricted to A24, B18, A2, B8, B35 and B57 and recognized epitopes dispersed throughout the 491-amino-acid IE protein^{16,18-20}, also failed to recognize IE if pp65 was co-expressed in target cells (data not shown). Moreover, pp65 did not interfere in co-infection experiments with presentation to CTL of a CMV envelope protein, gB, or a human immunodeficiency virus-1 structural protein, p55 Gag (data not shown). The effect of pp65 did not result from modification of IE protein expression or stability, because IE synthesis and degradation rates were equivalent in fibroblasts infected with either vac/IE or CMV alone, or co-infected with either vac/IE and vac/pp65 or vac/IE and vac/pp150 (Fig. 2a, b).

To determine whether the small amount of pp65 introduced with the CMV virion is sufficient to interfere with IE presentation, a pp65-deletion mutant of CMV strain AD169 (RVAd65) was constructed. This mutant contains a matrix devoid of pp65 but expresses IE in amounts comparable to wild-type CMV²¹. Fibroblasts were infected with wild-type and RVAd65 CMV virus in the

FIG. 1 a, Class-I-restricted presentation of IE protein in cells expressing IE alone or in the presence of other CMV proteins. Autologous fibroblasts were infected with either vac/IE, vac/pp150 or CMV alone or co-infected with vac/IE and CMV or vac/pp150 and CMV, and analysed for cell lysis by CTL clones specific for IE (TM2A3; black bars) or pp150 (TM3C11; grey bars) in a 5-h chromium-release assay¹¹. b, Class-I-restricted presentation of IE in the presence or absence of matrix proteins pp65 and pp150. Fibroblasts were co-infected with combinations of vaccinia viruses and analysed for cell lysis by CTL clones specific for IE (TM2A3; black bars), pp150 (TM3C11; light grey bars) or pp65 (CM7C3; dark grey bars)²². Data are presented at an effector:target (E:T) ratio of 10:1; similar results were obtained at an E:T ranging from 5:1 to 20:1.



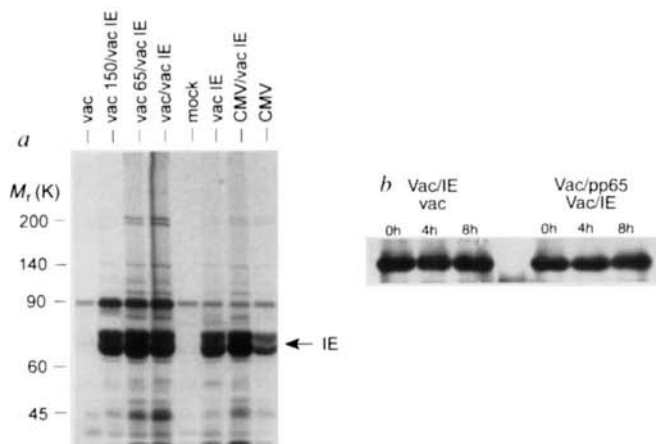


FIG. 2 **a**, IE protein expression in targets expressing IE alone or in the presence of other CMV proteins. Fibroblasts were infected with CMV and vaccinia viruses, radiolabelled with ^{35}S -methionine, immunoprecipitated with IE-specific antibody, and analysed for IE protein expression by SDS-PAGE. **b**, Comparison of IE degradation rate in the presence and absence of pp65. Cells were co-infected with either vac/IE and vac or vac/IE and vac/pp65, pulsed with ^{35}S -methionine, then incubated for the indicated times after addition of unlabelled methionine; cell lysates were then immunoprecipitated with antibody specific for IE and analysed by SDS-PAGE.

presence of metabolic inhibitors of translation and transcription added sequentially to permit introduction of viral matrix proteins but new expression of only IE genes products^{11,12}. IE-specific CTLs recognized targets infected with RVAd65 but did not lyse CMV-infected targets (Fig. 3). By contrast, CTLs specific for the introduced pp150 matrix protein lysed targets infected with either virus.

pp65 expression results in phosphorylation of several CMV proteins²¹, and pp65 isolated from infected cells phosphorylates *in vitro* the casein kinase II substrates phosphovitin and casein^{20,22,23}, so pp65 or a cellular kinase that interacts with pp65 might modify IE by phosphorylation. The carboxy terminus of pp65 contains conserved sequences of serine/threonine kinases²², and a recombinant vaccinia vector was constructed to contain a mutant pp65 (vac/ Δ 65) with 174 base pairs (bp) deleted, including the putative ATP-binding site and the invariant lysine required for phosphotransfer reaction²². After infection of cells with vac/ Δ 65, the immunoprecipitated mutant protein did not show any kinase activity *in vitro* (data not shown). Fibroblasts were co-infected with vac/IE and vac, vac/IE and vac/pp65, or vac/IE and vac/ Δ 65, and the expressed IE proteins immunoprecipitated. IE protein expression and tyrosine phosphorylation were similar in cells co-infected with vac/IE and either vac/pp65 or vac/ Δ 65 (Fig. 4a). No serine-phosphorylation of IE was detected in any of these cells (data not shown). However, expression of full-length pp65, but not Δ 65, resulted in increased threonine-phosphorylation of IE (Fig. 4a). These targets were also analysed for lysis by IE-specific CTL clones and by a CTL clone specific for an epitope on pp65 present in both the wild type and Δ 65 mutant. Co-infected targets expressing either pp65 or Δ 65 were lysed by pp65-specific CTLs, but the Δ 65 deletion mutant (in contrast to wild-type pp65) did not prevent IE presentation (Fig. 4b).

These results show that structural viral proteins released into the cytosol after virion entry can modify presentation of endogenously synthesized viral antigens, and that presentation of a viral protein can be selectively blocked at a processing step before peptide loading, without general blockade of class I antigen presentation in the infected cell. In the case of CMV, pp65 expression results in increased phosphorylation of IE in threonine residues and interferes with IE presentation without detectable changes in either the stability of the IE protein or its ability to

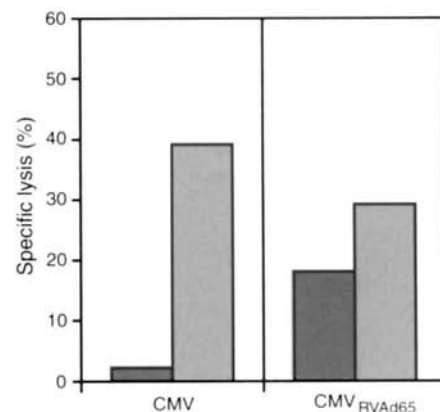


FIG. 3 IE-protein presentation in cells infected with a mutant CMV bearing a deletion in the pp65 gene. Fibroblasts were infected with either wild-type CMV or CMV RVAd65 lacking the *UL83* gene encoding pp65, under conditions in which metabolic inhibitors permit new transcription and translation of only viral IE genes, and then evaluated for lysis by CTLs specific for IE (dark grey bars) or pp150 (light grey bars) at E:T of 10:1.

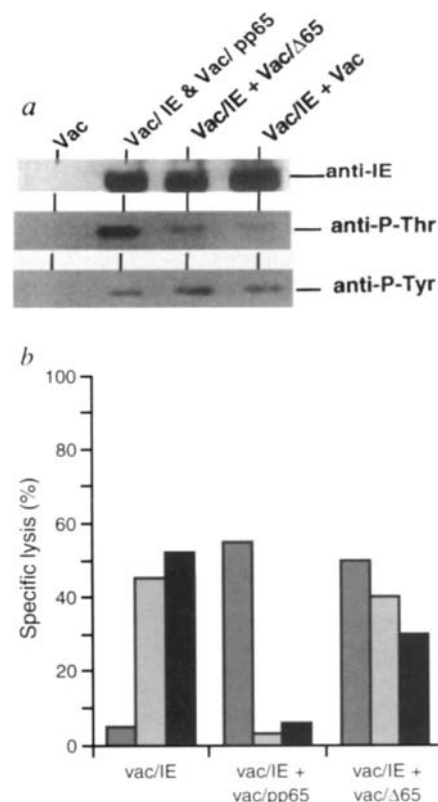


FIG. 4 **a**, Phosphorylation of IE protein in the presence and absence of pp65 and mutant pp65. Fibroblasts were infected with recombinant vaccinia viruses, immunoprecipitated with IE-specific antibody, transferred to nitrocellulose, and probed with antibodies specific for IE, phosphothreonine or phosphotyrosine. **b**, Presentation of IE in association with two class-I-restricting elements in cells co-expressing IE and either pp65 or Δ 65. Fibroblasts expressing HLA-A24 or HLA-B18 were co-infected with recombinant vaccinia viruses and analysed for lysis by CTLs specific for pp65 (CM7C3; dark grey bars) or CTLs specific for IE (TM2A3, light grey bars; MR3H9, black bars) in a standard chromium-release assay. Data are presented at an E:T of 10:1.

activate transcription^{21,24}. This suggests that phosphorylation of IE residues may either severely restrict access of the protein to the antigen-processing machinery or divert the protein to a different degradative pathway, providing an evasion strategy that could be crucial for CMV persistence. Similar modification of other viral or cellular proteins may regulate the access of protein antigens to processing/presentation for recognition by CTL. □

Methods

Viruses. CMV strain AD169 was obtained from the American Tissue Culture Collection²¹; the mutant AD169 strain, RVAd65, with a deleted *UL83* gene, was constructed by B. Plachter²¹. Vac/IE, a recombinant vaccinia virus encoding the major immediate-early protein of CMV strain AD169, was obtained from E. Paoletti^{11,12}. Vac/pp150, a recombinant vaccinia virus encoding the gene for CMV pp150 was constructed by homologous recombination between vaccinia strain WR and DNA from a plasmid, pSC11, encoding the full-length pp150 gene. Vac/pp65, a recombinant vaccinia virus encoding CMV pp65 of CMV strain AD169, was obtained from W. Britt. Vac/Δ65 contains a mutant pp65 deleted of a 174-base segment between base pairs 1,194 and 1,368, which was constructed by digestion first with *Afl*III, then mung-bean nuclease, then with *T*thIII, after which protruding ends were filled in using Klenow polymerase and blunt-end ligation of the 3'-coding sequence in-frame²⁵. The Δ65 mutant gene was inserted into vaccinia by homologous recombination^{11–14}.

T cells. T-cell clones specific for IE (TM2A3 restricted to HLA-A24 and MR3H9 restricted to HLA-B18), pp65 (CM7C3 restricted to HLA-B35) and pp150 (TM3C11 restricted to HLA-A24) were isolated from CMV-seropositive individuals as described^{11,12,15}. Cytolytic function was assessed by 5-hour chromium release assay using low-passage fibroblasts from donor M.R. (HLA: A24, 25; B18, 35), infected for 12 h with combinations of recombinant vaccinia viruses at a multiplicity of infection (MOI) of 10, CMV at MOI 5, or ΔCMV at MOI 25 (ref. 11).

Radioimmunoprecipitation. 10⁶ fibroblasts were either mock-infected, infected with CMV, or co-infected with CMV and vac/IE, vac/IE and vac, vac/IE and vac/pp65, or vac/IE and vac/pp150. After 12 h, cells were incubated with 200 μCi ³⁵S-methionine per 10⁶ cells for 30 min, detergent-lysed by Nonidet P-40, immunoprecipitated with an IE-specific monoclonal antibody 6E3 (ref. 26) and staphylococcal protein A cells, fractionated by SDS-PAGE, and analysed by autoradiography¹¹.

Pulse-chase experiments. Human fibroblasts (10⁶) were infected for 12 h with vac/IE and vac, or vac/IE and vac/pp65, and radiolabelled with ³⁵S-methionine. Following a 30-min pulse, infected fibroblasts were washed with Dulbecco's MEM medium with 10% fetal calf serum and unlabelled methionine and either lysed and immunoprecipitated with anti-IE monoclonal antibody (6E3)²⁶ (time point '0')¹¹ or incubated for a further 4 or 8 h before lysis and immunoprecipitation.

Sequential metabolic blockade of translation and transcription of CMV-infected cells. Fibroblasts were pretreated for 30 min with 50 μg ml⁻¹ cyclohexamide to block translation, infected with wild-type CMV (AD169) or mutant CMV lacking the *UL83* gene encoding pp65 (RVAd65)²¹ at MOI 25. Four hours after cyclohexamide exposure, cells were washed with PBS containing 100 μg ml⁻¹ actinomycin D to block transcription and then incubated for 3 h in medium containing actinomycin D to block transcription but permit translation of existing IE mRNA. Cells were washed twice with PBS containing actinomycin D before chromium-release assay¹².

Immunoblots for IE, phosphothreonine, phosphotyrosine and phosphoserine. 10⁶ late-passage fibroblasts were infected at MOI 10 with vac/IE and vac, vac/IE and vac/Δ65, vac/IE and vac/pp65, or with vac alone as control, incubated for 12 h, and then lysed and immunoprecipitated with the IE-specific monoclonal antibody (mAb) 810 (Chemicon) as already described. Samples were fractionated on 10% SDS-PAGE, transferred to nitrocellulose, and analysed by immunoblotting for IE protein and for phosphorylation on serine, threonine and tyrosine residues, with monoclonal antibodies 810, P-3555 (specific for phosphothreonine residues; Sigma), P-3300 (specific for phosphothreonine residues; Sigma), and P-3430 (specific for phosphoserine; Sigma). Blots were enhanced with goat anti-mouse secondary antibody (Biosource), and chemiluminescence reagent (NEN), and analysed by autoradiography.

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Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands

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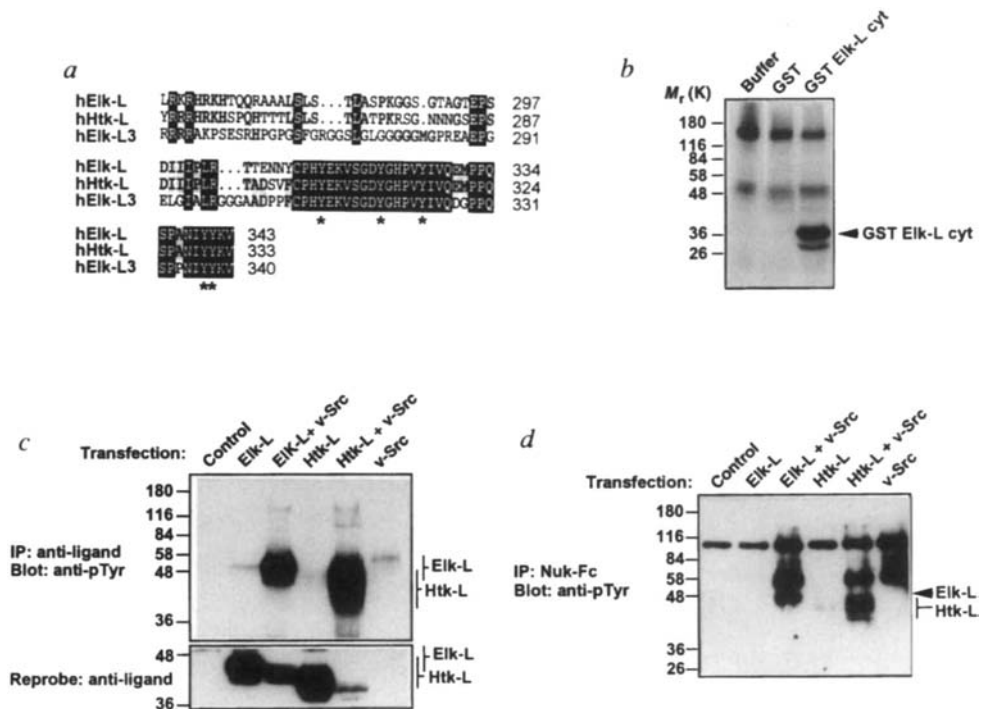
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RECEPTOR tyrosine kinases of the EPH class have been implicated in the control of axon guidance and fasciculation^{1–7}, in regulating cell migration⁸, and in defining compartments in the developing embryo^{9–11}. Efficient activation of EPH receptors generally requires that their ligands be anchored to the cell surface, either through a transmembrane (TM) region or a glycosyl phosphatidylinositol (GPI) group¹². These observations have suggested that EPH receptors can transduce signals initiated by direct cell–cell interaction. Genetic analysis of Nuk, a murine EPH receptor that binds TM ligands, has raised the possibility that these ligands might themselves have a signalling function⁶. Consistent with this, the three known TM ligands have a highly conserved cytoplasmic region, with multiple potential sites for tyrosine phosphorylation^{12–17}. Here we show that challenging cells that express the TM ligands Elk-L or Htk-L with the clustered ectodomain of Nuk induces phosphorylation of the ligands on tyrosine, a process that can be mimicked both *in vitro* and *in vivo* by an activated Src tyrosine kinase. Co-culture of cells expressing a TM ligand with cells expressing Nuk leads to tyrosine phosphorylation of both the ligand and Nuk. These results suggest that the TM ligands are associated with a tyrosine kinase, and are inducibly phosphorylated upon binding Nuk, in a fashion reminiscent of cytokine receptors¹⁸. Furthermore, we show that TM ligands, as well as Nuk, are phosphorylated on tyrosine in mouse embryos, indicating that this is a physiological process. EPH receptors and their TM ligands therefore mediate bidirectional cell signalling.

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FIG. 1 Phosphorylation of transmembrane ligands by v-Src. **a**, Alignment of human Elk-L, Htk-L and Elk-L3 cytoplasmic domains. Black and grey boxes indicate residues conserved in all or two TM-ligands, respectively; asterisk indicates conserved tyrosines. Elk-L is also referred to as Lerk-2 (ref. 13) and Cek5-L (ref. 14), Htk-L as Lerk-5 (ref. 26) and Elf-2 (ref. 15) and Elk-L3 as Nlerk-2 (ref. 29). **b**, Phosphorylation of GST-hElk-L cytoplasmic-domain fusion protein (GST-Elk-L cyt) *in vitro* by v-Src. v-Src was immunoprecipitated from v-src-transformed Rat-2 cells and incubated with GST fusion proteins in the presence of [γ - 32 P]ATP. **c**, **d**, Tyrosine phosphorylation of Elk-L and Htk-L upon coexpression with v-Src in Cos-1 cells. Ligands were immunoprecipitated with anti-ligand antibodies (**c**) or using Nuk-Fc as an affinity reagent (**d**), and immunoblotted with anti-phosphotyrosine antibodies. The band at ~100K in **d** represents cross-reaction of Nuk-Fc with protein A-HRP.



Nuk belongs to a subclass of EPH receptors that bind specifically to the TM subgroup of EPH-receptor ligands^{11,14,19}. Genetic analysis of *Nuk* has revealed a physiological requirement for this receptor for correct pathfinding of specific anterior commissure (AC) axons in the mouse brain⁶. Surprisingly, however, a truncated Nuk polypeptide containing the extracellular and TM domains, but lacking the kinase domain, supports normal AC formation in some genetic backgrounds. Nuk is primarily expressed in cells immediately ventral to the AC, whereas TM ligands are expressed in the commissural axons, raising the possibility that the TM ligands might themselves possess a signaling function, which is activated by binding of the Nuk extracellular domain⁶. The three known TM ligands have highly conserved cytoplasmic domains, and are virtually identical over their C-terminal 33 amino acids¹²⁻¹⁷. These sequences contain five potential tyrosine-phosphorylation sites²⁰ (Fig. 1a), suggesting that the TM ligands might signal in conjunction with a tyrosine kinase. Consistent with this, a glutathione *S*-transferase (GST) fusion protein containing the cytoplasmic domain of human Elk-L (residues 262–343) was tyrosine-phosphorylated by v-Src *in vitro*, whereas GST alone, or a fusion protein containing the Elk-L extracellular domain, was not (Fig. 1b, and data not shown).

To investigate whether full-length Elk-L or Htk-L could be phosphorylated on tyrosine *in vivo*, these TM ligands were expressed in Cos-1 cells either alone or in combination with v-Src. The ligands were then precipitated from the transfected cells using either an antibody against the common C-terminal region of Elk-L and Htk-L (anti-ligand) or a fusion protein containing the extracellular domain of Nuk fused to the Fc region of the immunoglobulin heavy chain (Nuk-Fc¹¹), which binds with high affinity to the extracellular domain of TM ligands. When these precipitates were blotted with ligand antibody, a diffuse band corresponding to a relative molecular mass (M_r) of ~45K–48K was specifically detected in cells transfected with Elk-L, whereas a protein of M_r between 38K and 46K was identified in cells transfected with Htk-L (Fig. 1c, lower panel). The predicted M_r for Elk-L is 38K and that for Htk-L is 37K, and their slow electrophoretic mobility is apparently due to glycosylation. Immunoblotting of anti-ligand immunoprecipitates from transfected Cos-1 cells with antibodies against phosphotyrosine (Fig. 1c, upper panel) showed that both Elk-L and Htk-L were

basally phosphorylated on tyrosine at low levels. Co-transfection of Elk-L with v-src led to the appearance of a highly tyrosine-phosphorylated ~50K form of Elk-L in both anti-ligand and Nuk-Fc precipitates (Fig. 1c, upper panel, and **d**). In addition, a tyrosine-phosphorylated protein of 130K–140K co-precipitated with Elk-L from cells coexpressing ligand and v-Src. v-Src also induced strong tyrosine phosphorylation of Htk-L, which migrated as a broad band of 38K–48K in the phosphorylated form. Immunoprecipitation of both Elk-L/Htk-L and the 130K protein was markedly reduced by addition of the immunizing peptide, which competes for antibody binding (data not shown). In v-src co-transfected cells, there was a reduction in the total amount of either ligand detected in western blots by anti-ligand antibodies. These antibodies, which were raised against the C-terminal part of Elk-L, may be less efficient in recognizing highly tyrosine-phosphorylated forms of the denatured ligands in an immunoblot. These results show that Elk-L and Htk-L are potent *in vivo* substrates for an activated Src tyrosine kinase and can be detected in association with other phosphotyrosine-containing proteins in cells expressing both ligand and v-Src.

The phosphorylation of TM ligands on tyrosine may provide a mechanism by which signals are transmitted into ligand-presenting cells. We reasoned that such a signal might be activated by the clustering of ligands on the cell surface. We therefore exposed Cos-1 cells expressing Elk-L or Htk-L to the Nuk extracellular domain, in the form of Nuk-Fc fusion protein clustered with anti-immunoglobulin. Under these conditions, Nuk-Fc induced a several-fold increase in tyrosine phosphorylation of both Elk-L and Htk-L, whereas no stimulation of ligand tyrosine phosphorylation was induced by Fc alone (Fig. 2a). The tyrosine-phosphorylated band immunoprecipitated by the anti-ligand serum was markedly reduced by addition of excess ligand C-terminal peptide to the immunoprecipitates. This experiment indicates that binding of clustered Nuk-Fc to the TM ligands activates an endogenous tyrosine kinase in Cos-1 cells which can subsequently phosphorylate Elk-L and Htk-L. These results were obtained using exogenously overexpressed ligand, however. To corroborate our observations in a physiologically relevant cell type, we used the human neuroepithelioma cell line CHP-100, which expresses endogenous Elk-L (ref. 12). Incubation of CHP-100 cells with clustered Nuk-Fc led to a striking increase in

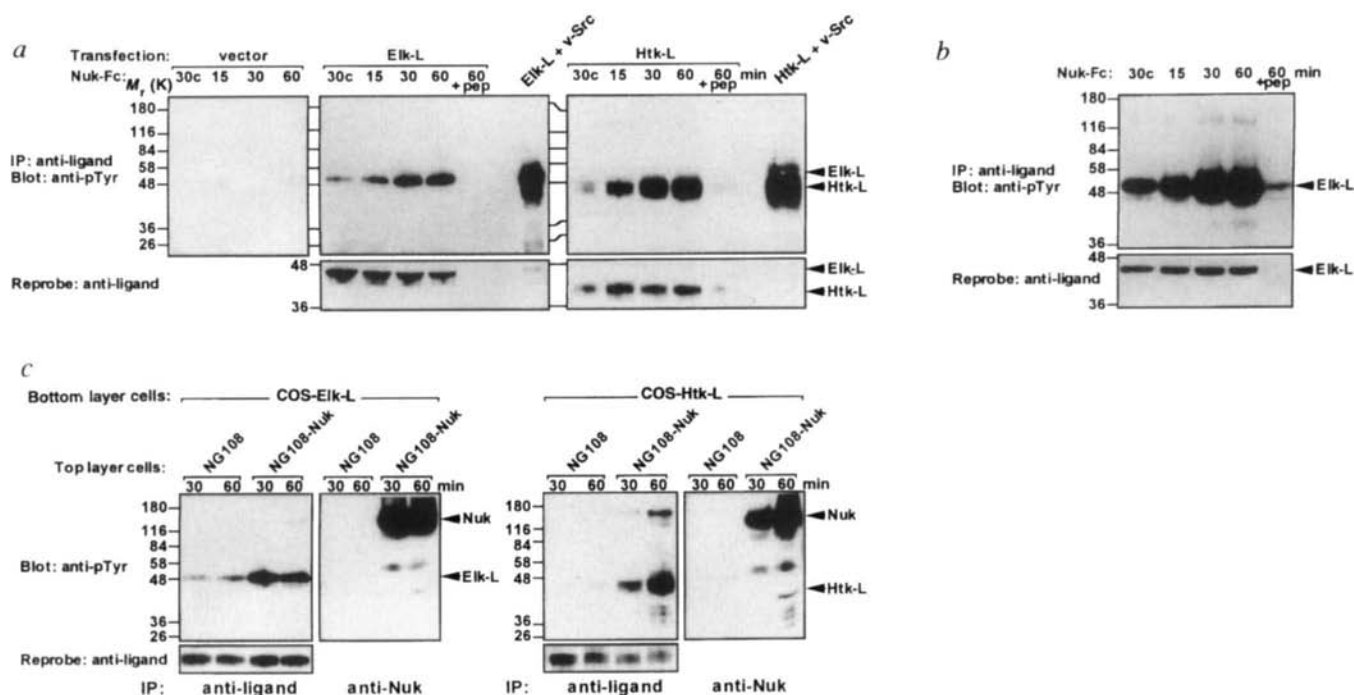


FIG. 2 Stimulation of tyrosine phosphorylation of TM ligands by Nuk extracellular domain and Nuk-expressing cells. *a*, Cos-1 cells were transiently transfected with Elk-L, Htk-L, or control expression vectors, and treated with $2 \mu\text{gml}^{-1}$ clustered Nuk-Fc or Fc (*c*) for the indicated periods (min). Ligands were immunoprecipitated (IP) with anti-ligand antibodies and blotted with anti-phosphotyrosine antibodies (top panels). Lower panels, anti-ligand blot (reprobe). '+ pep' Competing immunizing peptide was added. *b*, CHP-100 cells, which express endogenous Elk-L,

were stimulated and immunoblotted as for *a*. Top panel, anti-phosphotyrosine blot; lower panel, anti-ligand blot (reprobe). *c*, Bidirectional signaling between Nuk-expressing and TM-ligand-expressing cells in co-culture. Cos-1 cells transiently transfected with Elk-L or Htk-L were co-cultured with parental or Nuk-expressing NG108 cells for the indicated times. Left panels, anti-ligand immunoprecipitate; right panels, anti-Nuk IP from pooled, co-cultured cells. Top, anti-phosphotyrosine blots; bottom, anti-ligand blots (reprobe).

tyrosine phosphorylation of Elk-L and to co-precipitation of several tyrosine-phosphorylated polypeptides (Fig. 2*b*). Thus, the binding of the Nuk extracellular domain to a cell that normally expresses Elk-L also leads to tyrosine kinase activation and concomitant Elk-L phosphorylation.

These findings raised the possibility that interaction of a cell expressing TM ligands on its surface with a second cell expressing

Nuk might lead to the activation of the Nuk receptor, and subsequent signalling within the Nuk-expressing cell, and also to the activation of a ligand-associated kinase and consequent ligand phosphorylation. We therefore co-cultured Cos-1 cells expressing Elk-L or Htk-L with the neuronal cell line NG108-15 (ref. 21) (NG108), which lacks endogenous EPH receptors that bind TM ligands, or with a transfected NG108 clone that stably expresses high levels of the 130K mouse Nuk protein (NG108-Nuk). In co-cultures of ligand-expressing cells with NG108-Nuk cells, we observed Nuk tyrosine phosphorylation, reflecting activation of the Nuk catalytic domain, and also tyrosine phosphorylation of Elk-L or Htk-L, consistent with stimulation of a ligand-associated tyrosine kinase in the ligand-expressing cells (Fig. 2*c*). Parental NG108 cells lacking Nuk were without effect and conversely, no phosphorylation of either TM ligands or Nuk was induced using untransfected Cos-1 cells (Fig. 2*c*, and data not shown).

The observation that Elk-L and Htk-L are inducibly phosphorylated on tyrosine in cultured cells on exposure to clustered Nuk-Fc or Nuk-expressing cells suggests that this may be a physiological event. We therefore immunoprecipitated protein lysates from mouse embryos at 10.5 days of development (E10.5) with antibodies against either TM ligands or Nuk, and immunoblotted the immune complexes with antibodies against phosphotyrosine (Fig. 3). Nuk immunoprecipitated from embryonic body or head tissue was phosphorylated on tyrosine (Fig. 3, and data not shown). Furthermore, anti-ligand antibodies specifically precipitated phosphotyrosine-containing polypeptides from these embryonic lysates that co-migrated with v-Src-phosphorylated TM ligands. The intensity of the tyrosine-phosphorylated band immunoprecipitated by the anti-ligand antibodies was markedly reduced by addition of excess ligand C-terminal peptide. These results show that it is not only EPH receptors such as Nuk but also

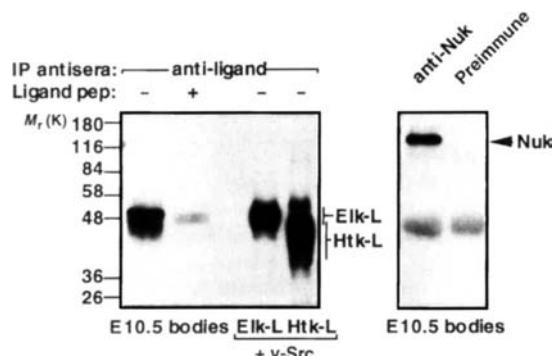
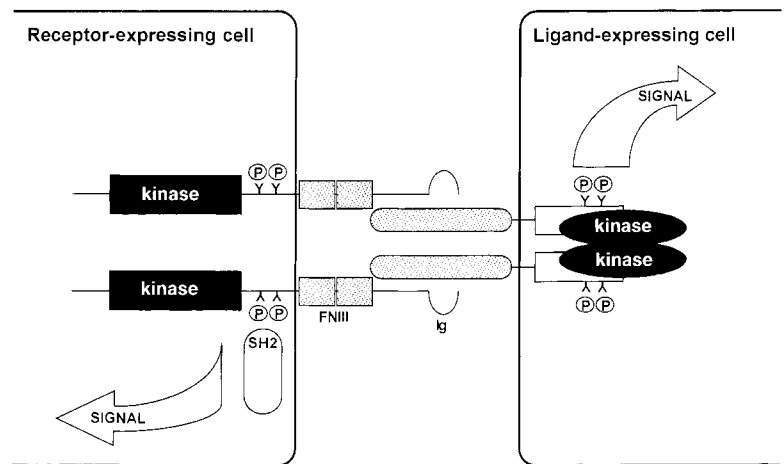


FIG. 3 Transmembrane ligands and Nuk are phosphorylated on tyrosine in the mouse embryo. Anti-phosphotyrosine immunoblot of TM ligands (left) and Nuk receptor (right) immunoprecipitated from E10.5 mouse body tissue. Lysed tissue was immunoprecipitated with anti-Nuk or pre-immune serum, or anti-ligand antibodies with or without addition of excess competing immunizing peptide (ligand pep). Results for E10.5 head tissue were essentially identical. The mobility of tyrosine-phosphorylated Elk-L and Htk-L is indicated by inclusion of v-Src-phosphorylated TM ligands on the gel (Elk-L + v-Src and Htk-L + v-Src).

FIG. 4 Proposed model for bidirectional signalling by Nuk and its TM ligands. Interaction of the ligand-expressing cell (right) with the receptor-expressing cell (left) promotes aggregation and autophosphorylation of the receptor. This is followed by recruitment of SH2-domain-containing proteins to phosphorylated tyrosines, for example in the juxtamembrane region^{27,28}, and tyrosine-phosphorylation of cellular proteins. Concomitantly, interaction of the receptor with TM ligands causes ligand clustering and phosphorylation by an associated tyrosine kinase, possibly leading to propagation of signals in the ligand-presenting cell.



their TM ligands that are phosphorylated on tyrosine in the developing mouse embryo.

EPH receptors have been implicated in establishing boundaries between two distinct cell types, for example in the rhombomeres of the hindbrain and in development of the forebrain²⁻¹¹. It would therefore be advantageous if cell-cell contact initiated a bidirectional signal thereby regulating the phenotype of both receptor- and ligand-expressing cells. We have proposed a biochemical mechanism for achieving such bidirectional signalling (Fig. 4). In the neuronal cell line NG108, activation of Nuk by TM ligands leads not only to Nuk autophosphorylation, but also to phosphorylation of potential receptor targets (Fig. 2c, and S.J.H. *et al.*, manuscript in preparation). Our data also indicate that binding of Nuk to TM ligands activates a tyrosine kinase in the ligand-expressing cell, leading to phosphorylation of the conserved C-terminal region of the ligand itself. One scheme consistent with our results is that phosphorylation of the ligands by Src-like kinases induces the binding of SH2-containing proteins which then transmit signals within the ligand-expressing cell; alternatively, ligand phosphorylation might modulate interactions with the cytoskeleton. However, the identities of the tyrosine kinases which phosphorylate TM ligands *in vivo* and the signalling proteins which bind activated ligands are not known. EPH family tyrosine kinases therefore function not only as receptors but also as activating ligands for transmembrane proteins such as Elk-L, Htk-L and presumably the recently cloned Elk-L3 (ref. 17). Interactions of this type may represent a general and economic mechanism by which cellular behaviour is controlled during embryonic development and axonal migration²²⁻²⁴. □

Methods

Reagents. Anti-ligand antibodies (Santa Cruz) were raised against residues 326–343 of hElk-L, and also recognize Htk-L. Anti-Nuk serum was described previously². Monoclonal anti-v-Src antibodies were from Oncogene Science. GST–Elk-L cyt contains the whole cytoplasmic domain (residues 262–343) of hElk-L (ref. 12) cloned into pGEX4T2.

Transfection and cell stimulation. Cos-1 cells were transiently transfected with 5–10 µg DNA using lipofectin reagent (Gibco). Cells were collected ~60 h post-transfection after 20 h in medium containing 0.5% fetal bovine serum. Human neuroepithelial CHP-100 cells were serum-starved for 8 h before stimulation. Nuk-Fc¹¹ and Fc were clustered using anti-human IgG (Jackson ImmunoResearch) for 1–2 h at 4°C and diluted to 2 µg ml⁻¹ in serum-free medium before applying to cells.

Co-cultures. NG108-15 cells (NG108: mouse neuroblastoma × rat glioma fusion²¹) were stably transfected with an expression vector containing full-length Nuk, and individual G418-resistant clones isolated (NG108-Nuk cells). Parental or Nuk-expressing NG108 cells were removed from the plate by titration and resuspended in PBS plus magnesium and calcium. Cell suspensions were placed on top of serum-starved Cos-1 cells transiently expressing TM ligands

and co-cultured for 30 or 60 min at 37°C with 5% CO₂. Pooled cells were lysed and lysates divided into two for immunoprecipitation.

Immunoprecipitation and immunoblotting. Cells were lysed in PLC lysis buffer². Lysates of E10.5 embryos from natural matings were prepared as described² and precleared by incubation with protein A–Sepharose. Immunoprecipitations were done using 1 µg anti-ligand antibodies, 5 µl anti-Nuk serum or 10 µg of Nuk-Fc fusion protein with protein A–Sepharose, or 1 µg monoclonal anti-v-Src antibodies with anti-mouse agarose for 1–2 h, then washed three times with HNTG buffer². Protein complexes were separated by 10% SDS–PAGE transferred to an Immobilon-P membrane (Millipore) and immunoblotted. Anti-phosphotyrosine blots were performed using 4G10 (UBI), except for Fig. 1d, for which polyclonal anti-phosphotyrosine antibodies were used. Membranes were stripped with 0.1 M glycine, pH 2.5, where indicated. Peptide competition with immunizing peptide (Santa Cruz) used approximately 100-fold excess peptide.

In vitro kinase assay. v-Src was immunoprecipitated from v-src-transformed Rat-2 cells and incubated for 10 min at room temperature with 5 µCi of [³²P]ATP in Src-kinase reaction buffer²⁵, or buffer containing 10 µg purified GST or GST–Elk-L cyt as exogenous substrate. Products were separated and ³²P-labelled proteins detected by autoradiography.

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Functional interactions between Stat5 and the glucocorticoid receptor

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SIGNAL transduction pathways enable extracellular signals to activate latent transcription factors in the cytoplasm of cells. Dimerization, nuclear localization and binding to specific DNA sequences result in the induction of gene transcription by these proteins. These events are necessary for the functioning of the JAK/STAT pathway and of the glucocorticoid-receptor pathway. In the former, the protein Stat5, which is a member of a family of signal transducers and activators of transcription, is activated by cytokines, hormones and growth factors¹⁻⁷. These polypeptide ligands bind at the outside of the cell to specific transmembrane receptors and activate intracellular Janus protein tyrosine kinases (JAKs) to tyrosine-phosphorylate STAT proteins; interaction with the SH2 domain of the dimerization partner then confers the ability to bind to DNA at the STAT-response element and induce transcription⁸⁻¹⁰. In the glucocorticoid-receptor pathway, the receptor interacts with its steroid hormone ligand in the cytoplasm, undergoes an allosteric change that enables the hormone receptor complex to bind to specific DNA-response elements (glucocorticoid response elements, or GRE) and modulate transcription^{11,12}. Although these pathways appear to be unrelated, we show here that the glucocorticoid receptor can act as a transcriptional co-activator for Stat5 and enhance Stat5-dependent transcription. Stat5 forms a complex with the glucocorticoid receptor which binds to DNA independently of the GRE. This complex formation between Stat5 and the glucocorticoid receptor diminishes the glucocorticoid response of a GRE-containing promoter.

We used a COS cell co-transfection assay to study the hormonal synergism between prolactin and glucocorticoid hormones. COS cells do not express endogenous prolactin receptor or Stat5, a factor that confers the response to prolactin¹³⁻¹⁵, and only trace amounts of glucocorticoid receptor are present¹⁶. We introduced a glucocorticoid receptor gene together with Stat5, the prolactin receptor¹⁷, and a β -casein-gene-promoter luciferase construct into COS cells (Fig. 1). Induction of the cells with prolactin resulted in ~10-fold increase in luciferase activity over untreated cells (lanes 2 and 5), confirming previous results^{15,18,19}. Glucocorticoid hormone treatment had either no or only a very minor effect (lane 3). Simultaneous induction with prolactin and glucocorticoid hormone increased the luciferase activity ~40-fold (lane 4). This experiment shows that the synergism between prolactin and glucocorticoid hormone action, well documented in mammary epithelial cells²⁰⁻²², can be reconstituted in transfected COS cells.

The β -casein promoter element used in the transfection experiments (Fig. 1) is either not or only very weakly affected by the activated glucocorticoid receptor. It comprises binding sites for several nuclear factors²³ but does not contain a GRE consensus sequence²³. To investigate the mechanism of synergism in transcriptional induction between Stat5 and the glucocorticoid receptor, we tested for complex formation in a co-immunoprecipitation experiment (Fig. 2). COS cells were transfected with Stat5, the prolactin receptor, and the glucocorticoid-receptor genes, and induced with prolactin and glucocorticoid hormone. Nuclear extracts were prepared and immunoprecipitated with a Stat5-specific antiserum; immunoprecipitates were developed on western blots with a glucocorticoid-receptor-specific antiserum (Fig. 2a). The glucocorticoid receptor can be co-precipitated with Stat5

in cells induced by dexamethasone and prolactin (lane 4), indicating that a direct protein-protein interaction, independent of DNA binding, occurs between both transcription factors in the induced state. The result was consistent when the glucocorticoid-receptor-specific antiserum was used for immunoprecipitation and the Stat5-specific antiserum for western blotting (Fig. 2b).

Complex formation between Stat5 and the glucocorticoid receptor was confirmed in electrophoretic mobility shift assays (EMSA). A protein-DNA complex induced by treatment of COS cells transfected with mammary gland factor (MGF)-Stat5 and glucocorticoid-receptor genes and treated with prolactin and dexamethasone (Fig. 2c, lanes 10-12) could be supershifted with an antiserum specific for Stat5 (lane 11) or glucocorticoid receptor (lane 12). Nuclear extracts from cells transfected with MGF-Stat5 (lanes 1-4) or glucocorticoid receptor genes (lanes 5-8) served as controls.

The mouse mammary-tumour virus long terminal repeat (MMTV-LTR) contains a promoter region with several glucocorticoid-receptor binding sites (GRE) and responds to induction by glucocorticoid hormones^{24,25}. Introduction of a MMTV-LTR luciferase gene and the glucocorticoid receptor into COS cells results in a strong inducibility by glucocorticoid hormone (Fig. 3, lane 2). Simultaneous introduction of Stat5 and activation of Stat5 by prolactin strongly suppresses the response of the MMTV-LTR towards glucocorticoids (Fig. 3, lane 3). This effect is not just due to the presence of Stat5, but requires Stat5 activation through the prolactin receptor (Fig. 3, lane 4).

The experiments described shed light on the synergistic action between Stat5 and the glucocorticoid receptor in the induction of the β -casein gene in mammary epithelial cells. Stat5 and glucocorticoid receptor form a molecular complex and cooperate in transcriptional induction. We believe that the strong transactivation domain of the glucocorticoid receptor enhances Stat5 action—the transactivation domain of Stat5 by itself is relatively weak²⁶. The complex formation between Stat5 and the glucocorticoid receptor is an interesting example of cooperation between apparently unrelated signalling pathways. In the presence of glucocorticoid hormone, only the glucocorticoid receptor is activated to induce

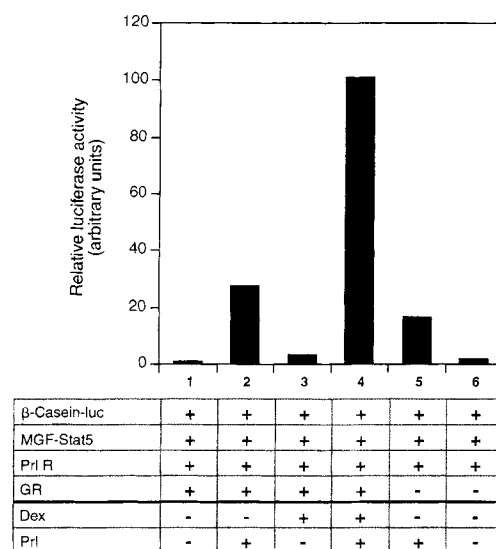


FIG. 1 Stat5-mediated prolactin induction of the β -casein gene promoter is enhanced by glucocorticoid-receptor activation. COS7 cells were transfected with constructs encoding Stat5 (MGF-Stat5), the prolactin receptor (PrlR), the glucocorticoid receptor (GR) and a β -casein promoter for the luciferase gene (β -casein-luc). Luciferase activity was measured after treatment of the cells with no hormone (lane 1), prolactin (Prl, lane 2), dexamethasone (Dex, lane 3), dexamethasone and prolactin (lane 4). Cells in which the glucocorticoid receptor has been omitted from the transfection and which were treated with prolactin (lane 5) or no hormone (lane 6) were used as controls.

FIG. 2 Stat5 and glucocorticoid receptor from a molecular complex. **a** and **b**, COS7 cells were transfected with constructs encoding Stat5, glucocorticoid receptor and prolactin receptor. Cells were induced with no hormones (lane 1), dexamethasone (lane 2), prolactin (lane 3) or dexamethasone and prolactin (lane 4). Nuclear extracts were prepared from the induced cells and immunoprecipitated (IP) with Stat5-specific antiserum (**a**) or with glucocorticoid-specific antiserum (**b**). Immunoprecipitates were separated by SDS-PAGE and western blotted (WB) with a glucocorticoid-specific antiserum (**a**) or with Stat5-specific antiserum (**b**). Cells in which either glucocorticoid receptor (lanes 5 and 6 in **a**) or Stat5 had been omitted from the transfections (lanes 7 and 8 in **a**) were used as controls. Extracts from COS cells transfected with glucocorticoid receptor (lane C in **a**) or Stat5 (lane C in **b**) served as a control for the western blot. **c**, Nuclear extracts were prepared from cells transfected with MGF-Stat5 (lanes 1–4), with glucocorticoid receptor (lanes 5–8), and with MGF-Stat5 and glucocorticoid receptor (lanes 9–12). Cells shown in lanes 2–4 were induced with prolactin, in lanes 6–8 with dexamethasone, and in lanes 10–12 with prolactin and dexamethasone. EMSA was done as described³. Stat5-specific antiserum was included in the DNA-binding reaction shown in lanes 3, 8 and 11; glucocorticoid-receptor-specific antiserum was included in the DNA-binding reaction shown in lanes 4, 7 and 12. PR, PrR (prolactin receptor).

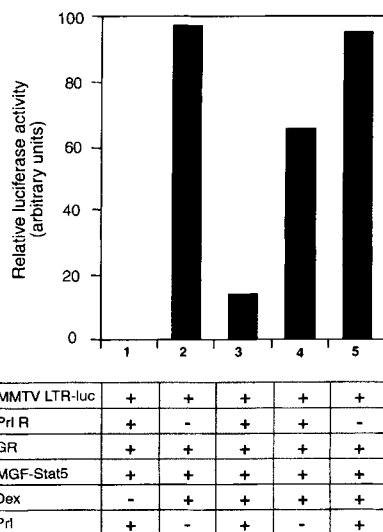
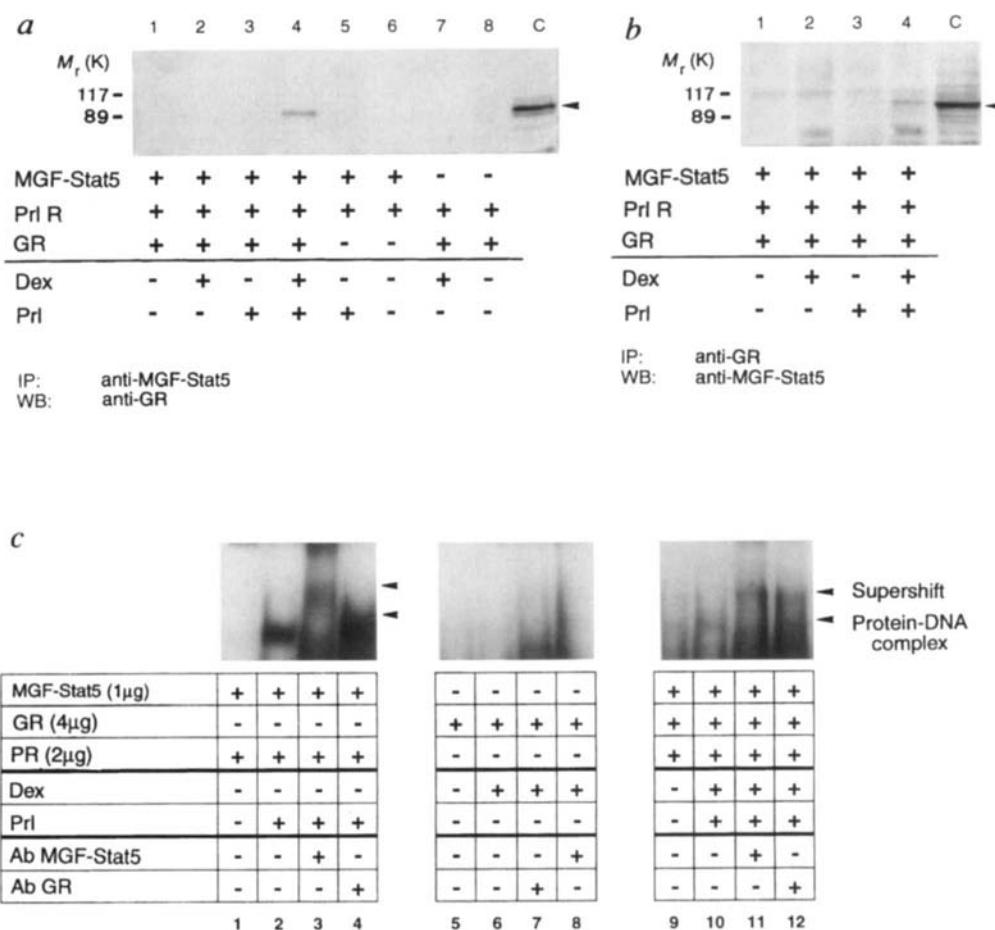


FIG. 3 Complex formation between Stat5 and the glucocorticoid receptor suppresses glucocorticoid induction of the MMTV-LTR promoter. COS 7 cells were transfected with constructs encoding a MMTV-LTR luciferase, the prolactin receptor, the glucocorticoid receptor and Stat5. Cells were induced with prolactin (lane 1), dexamethasone (lane 2), or dexamethasone and prolactin (lane 3). Cells in which the prolactin receptor has been omitted from the transfection were used as controls (lanes 4 and 5).

GRE-containing promoters; prolactin by itself weakly activates the Stat5-binding-site-containing promoters. In the presence of both signals, a molecular switch downregulates GRE-containing promoters and upregulates Stat5-response element-containing promoters. As cytokines can mediate inhibition of glucocorticoid-induced apoptosis of lymphocytes²⁷, it may be that Stat-mediated suppression of gene induction by the glucocorticoid receptor is responsible for this anti-apoptotic effect.

It has been proposed that the glucocorticoid receptor may act differently in its role as a negative transcriptional regulator in its interactions with AP-1 (ref. 28) and NF- κ B (ref. 29). Our results define a new function for the glucocorticoid receptor as a ligand-dependent co-activator that is independent of the specific DNA-binding domain of the receptor. The synergism between Stat5 and glucocorticoid receptor survives in glucocorticoid-receptor variants lacking a GRE-binding function. The integrity of the Stat5-response element in the β -casein gene promoter and the activation of Stat5 through phosphorylation of a tyrosine residue at position 694 are prerequisites for the synergistic action of Stat5 and the glucocorticoid receptor (data not shown). There may be different co-activators for Stat5 that depend on individual cytokine signals and cell types and contribute to the signalling specificity³⁰. Such a model could explain the versatility of Stat5 in different cytokine signalling pathways. □

Methods

Cell culture and transfection. Transfection experiments were done using the calcium phosphate precipitation technique. Half-confluent COS7 cells in 10-cm

dishes were transfected with 2 µg prolactin receptor expression vector, 2 µg glucocorticoid receptor expression vector, 2.5 µg Stat5 (pXM-MGF/Stat5). In experiments with reporter assays, 2 µg of the (–344/–1) β-casein gene promoter or 2 µg of the MMTV-LTR luciferase gene construct were used. In addition, 2 µg plasmid pCH110 encoding the β-galactosidase gene were included in each transfection as an internal control for transfection efficiency. Where indicated, the transfected cells were treated with 10^{–7} M dexamethasone and/or 5 µg ml^{–1} ovine prolactin for 16 h before collection for luciferase assays or for 1 h for complex formation assays.

Luciferase and β-galactosidase assays. Luciferase was assayed as described²⁸ in three independent experiments.

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA). Subconfluent COS7 cells in 10-cm dishes were transfected and induced for 1 h before collection. Nuclear extracts were prepared¹⁸. Electrophoretic mobility shift assay and supershifts are described in ref. 3.

Co-immunoprecipitation assays. Nuclear extracts of co-transfected and prolactin- and dexamethasone-induced cells were prepared. The cleared supernatants were adjusted to 100 mM NaCl. Immunoprecipitations were carried out using polyclonal anti-Stat5a antibody²⁹ or glucocorticoid-receptor-specific antiserum¹⁶ and immunoprecipitates isolated on protein A–Sepharose. Bound proteins were analysed by immunoblotting with polyclonal antiserum directed against either the glucocorticoid receptor or Stat5.

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An oxysterol signalling pathway mediated by the nuclear receptor LXRα

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CHOLESTEROL and its oxysterol congeners are important constituents of cell membranes and function as intermediates in several crucial biosynthetic pathways. These compounds autoregulate their metabolic fate by end-product repression and activation of downstream catabolism¹. Although end-product repression by oxysterols is relatively well understood², the mechanism by which these compounds act as positive transcription signalling molecules is unknown. Here we identify a specific group of endogenous oxysterols that activate transcription through the nuclear receptor LXRα. Transactivation of LXRα by oxysterols occurs at concentrations at which these compounds exist *in vivo*. The most potent activators also serve as intermediary substrates in the rate-limiting steps of three important metabolic pathways: steroid hormone biosynthesis, bile acid synthesis, and conversion of lanosterol to cholesterol. Our results demonstrate the existence of a nuclear receptor signalling pathway for oxysterols and suggest that LXRα may be important as a sensor of cholesterol metabolites.

Human LXRα is an orphan member of the nuclear receptor superfamily, which has the potential to function as a ligand-dependent transcription factor when complexed with its heterodimeric partner, the retinoid-X receptor (RXR)³. To identify LXRα ligands, we prepared concentrated lipid extracts from a variety of tissues and tested for ability to activate LXRα in a high-throughput cotransfection assay similar to that used to identify

ligands for other receptors^{4,5}. For the initial screening, a chimaeric receptor was used in which the ligand-binding domain of LXRα was fused to the DNA-binding domain of the yeast transcription factor GAL4 (ref. 3). The resultant GAL4-LXRα expression plasmid was cotransfected together with a GAL4-responsive luciferase reporter plasmid into CV-1 cells and challenged with concentrates from several tissue sources. A significant (six-fold) induction of luciferase activity was seen with lipid extracts derived from breeding-bull testis (Fig. 1a). The migration of this lipid activity on reverse-phase high-pressure liquid chromatography (HPLC) (data not shown) indicates that the compound may be related to a class of meiosis-activating sterols (MAS) isolated from gonads⁶. To explore the possibility that these sterols were LXRα

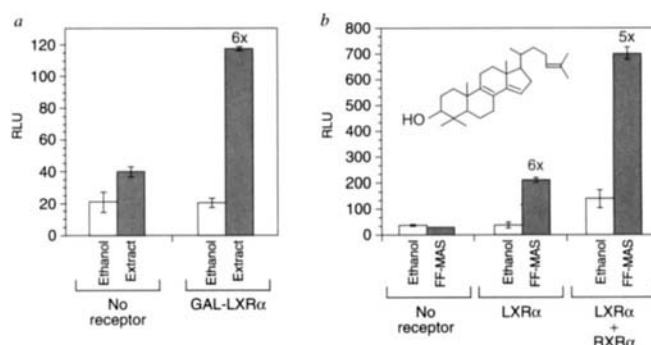
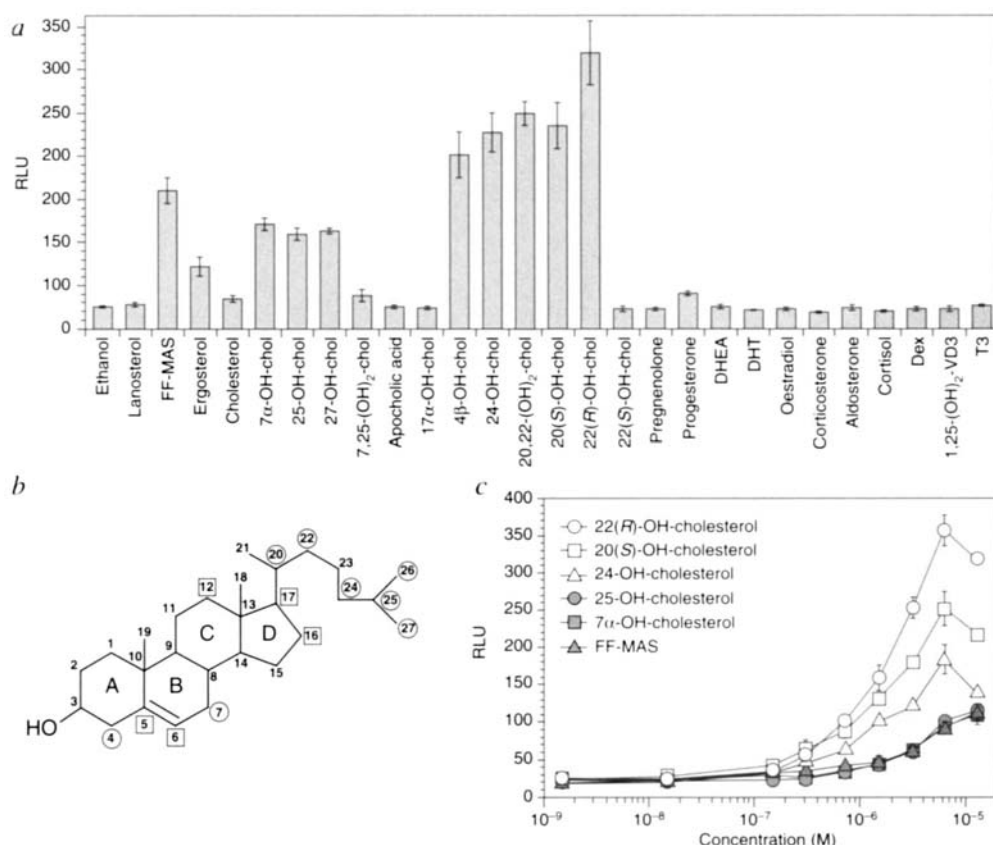


FIG. 1 Human LXRα is activated by gonad-specific sterols. **a**, Transactivation of LXRα ligand-binding domain (LBD) with testis extract. CV-1 cells were cotransfected with a GAL4-responsive luciferase reporter plasmid and an expression plasmid encoding no receptor or a chimaeric receptor composed of the GAL4 DNA-binding domain fused to the LXRα LBD (GAL4-LXRα)³. After transfection, cells were treated with ethanol or 1% of the concentrated testis extract. **b**, Transactivation of LXRα with follicular-fluid meiosis-activating sterol (FF-MAS). CV-1 cells were cotransfected with an LXRα-responsive reporter plasmid and the receptor expression plasmids for LXRα alone or with RXRα. After transfection, cells were treated with ethanol or 50 µM FF-MAS. Inset, structure of FF-MAS. RLU, Relative light units.

FIG. 2 LXR α is activated by a specific subset of oxysterols. **a**, Specificity of LXR α activators. Shown is a representative group from 70 compounds evaluated for LXR α activity (10 μ M) in CV-1 cell cotransfection assays. In addition to the compounds shown, farnesol, fatty acids and lanosterol precursors had no LXR α activity. DHEA, Dehydroepiandrosterone; DHT, 5 α -dihydrotestosterone; Dex, dexamethasone. **b**, Structure-activity relationship of LXR α activators. Data compiled from **a** and experiments not shown reveal that the position of the hydroxyl on cholesterol backbone is a determinant of LXR α activity. Circles and squares represent positions at which hydroxyl groups render the compound active or inactive, respectively. **c**, Dose responses for LXR α activators generated in CV-1 cell cotransfection assays. EC₅₀ values for LXR α activators are 1.5 μ M (22(R)-HC), 1.6 μ M (20(S)-HC), 1.6 μ M (24-HC) and 3.5 μ M (25-HC, 7 β -HC and FF-MAS).



activators, one of these compounds, FF-MAS (Fig. 1b, inset), was synthesized *de novo* and tested in the cotransfection assay using wild-type LXR α and a luciferase reporter plasmid containing the LXR-response element (TK-LXRE $\times$ 3-LUC)³. As in the results from testis extracts, a 5–6-fold induction of transcription by LXR α was seen in the presence of FF-MAS (Fig. 1b). Expression of RXR α above the endogenous level in CV-1 cells results in an enhancement of the LXR α response, consistent with the finding that LXR α and RXR α form an obligate heterodimer³.

In addition to regulating meiosis, FF-MAS is a biosynthetic precursor to cholesterol, and its ability specifically to induce LXR α transactivation prompted us to explore the possibility that related compounds in the cholesterol metabolic pathway might also activate LXR α . Over 70 compounds were tested, including the known nuclear receptor ligands and several intermediates in the biosynthetic pathways leading to cholesterol, steroid hormones and bile acids. Remarkably, only a specific group of oxysterols activated (5–15-fold) LXR α (Fig. 2a). The structure-activity relationships of these compounds reveal a requirement for the 3 β -hydroxyl group of cholesterol and an additional hydroxyl group preferentially located on the side chain of the molecule (Fig. 2b). The strongest LXR α activator is a naturally occurring compound, 22(R)-hydroxycholesterol (22(R)-HC). Significantly, the *S* enantiomer of this molecule (22(S)-HC) is completely inactive (Fig. 2a). Thus, both the precise location and the stereochemistry of the hydroxyl are important for activity. The position of the second hydroxyl group allows a distinct rank order of potency to be assigned: (22(R)-HC > 20(S)-HC > 24-HC > 25-HC = 7 α -HC = FF-MAS), with 22(R)-HC giving the most potent, saturable response (half-maximal effective concentration, EC₅₀ = 1.5 μ M; Fig. 2c). The concentrations at which these sterols elicit an LXR α response are within their known physiological range^{7–10}. Furthermore, these concentrations are at or below those required for ligand-dependent activation of other nuclear receptors (for example, FXR and PPAR)^{11–13} and the sterol-mediated repression of transcription modulated by sterol-

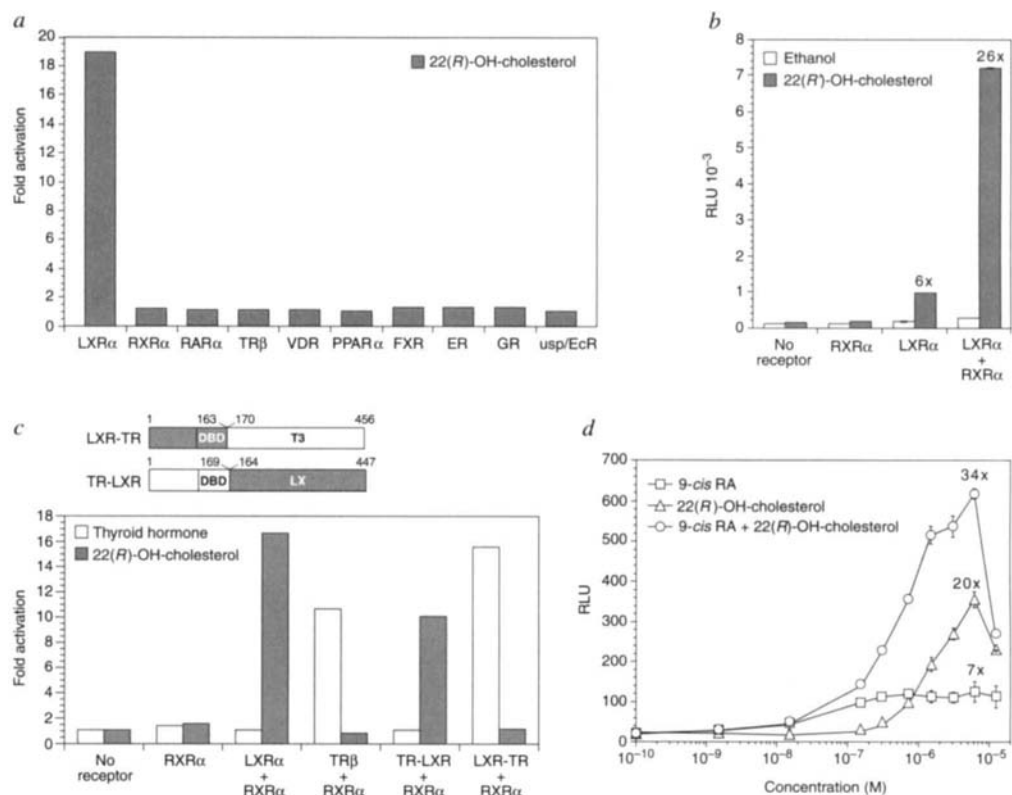
regulatory-element-binding proteins (SREBPs)². Our observations are strong evidence that these sterols may function as physiologically relevant activators of LXR α .

The unique structure-activity relationships for the LXR α activators described here are a hallmark of a receptor-mediated response. Consistent with this, transactivation by one of the most potent activators, 22(R)-HC, is LXR α -specific and shows no crossreactivity with a variety of other known nuclear receptors (Fig. 3a). This activity requires both LXR α and its response element, and is not observed on response elements of other nuclear receptors (data not shown). Furthermore, we note that the closely related LXR α subfamily member, referred to as UR (LXR β)¹⁴, is also activated by oxysterols (not shown). These results suggest the existence of a subfamily of nuclear oxysterol receptors.

To confirm that the oxysterol response is LXR α -dependent, we investigated whether this pathway could be recapitulated in a heterologous system. The insect cell line SL-2, which lacks mammalian nuclear receptors and is deficient in the metabolic pathways for cholesterol and bile acid synthesis¹⁵, was used as a transfection host in these experiments. These cells contain ultra-spiracle¹⁶, an RXR homologue that can form a functional heterodimer with LXR α on its response element (data not shown). Consequently, transfection of LXR α alone into SL-2 cells results in a six-fold induction by 22(R)-HC (Fig. 3b). As expected, when RXR α is cotransfected with LXR α , a robust (26-fold) increase in 22(R)-HC induction occurs (Fig. 3b). Taken together, these data support the hypothesis that LXR α directly mediates the 22(R)-HC transcriptional response.

One characteristic of all ligand-activated nuclear receptors is the presence of a functionally transferable ligand-binding domain¹⁷. To test for the existence of such a domain in LXR α that is responsive to 22(R)-HC, we expressed two chimaeric receptors (TR-LXR and LXR-TR) in which the ligand-binding domain of the thyroid hormone receptor (TR β) and the corresponding region of LXR α were exchanged (Fig. 3c). TR β was

FIG. 3 Activation by 22(R)-HC is LXR α -specific and occurs in a ligand-dependent manner. **a**, Receptor-specific transactivation by 22(R)-HC. CV-1 cells cotransfected with various nuclear receptors¹⁷ and their cognate luciferase reporters (see Methods) were treated with 6 μ M 22(R)-HC. **b**, Activation of LXR α by 22(R)-HC (10 μ M) can be reconstituted in transfected *Drosophila* SL-2 cells. **c**, Responsiveness to 22(R)-HC is mediated through the ligand-binding domain of LXR α . LXR-TR and TR-LXR chimaeric receptors used in these experiments are shown. CV-1 cells cotransfected with reporter and the indicated receptor combinations were treated with T₃ or 22(R)-HC. **d**, The RXR/LXR heterodimer is synergistically activated by 9-*cis* retinoic acid (9-*cis* RA) and 22(R)-HC. CV-1 cells cotransfected with LXR α receptor and reporter were treated with 9-*cis* RA, 22(R)-HC or both.



chosen for these studies because both TR β and LXR α can bind and transactivate the same response element (that is, the LXRE)³. As RXR heterodimers, LXR α and TR β respond to their cognate ligands (Fig. 3c). However, when the amino terminus and DNA-binding domain of TR β are fused to the putative ligand-binding domain of LXR α , the resultant TR-LXR chimera now responds to 22(R)-HC, but not thyroid hormone (Fig. 3c). As expected, the reciprocal chimera LXR-TR is not responsive to 22(R)-HC, but is responsive to thyroid hormone. These experiments show that the ligand-binding domain of LXR α is required for sterol responsiveness and that this region alone can transfer sterol inducibility to another protein, further supporting the proposal that LXR α is a sterol-responsive receptor.

A subset of receptors that function as RXR heterodimers have the unique ability to be activated by their own ligand, the RXR ligand (9-*cis* retinoic acid), or both ligands together^{11,18}. The RXR/LXR heterodimer falls into this category of receptors as this heterodimer can be activated by 9-*cis* retinoic acid or 22(R)-HC in a dose-dependent manner, with maximal inductions of 7-fold and 20-fold, respectively (Fig. 3d). Significantly, even at suboptimal concentrations, activation by both compounds together is more than additive, achieving a maximum induction of >30-fold. These results are consistent with the hypothesis that each receptor

within the RXR/LXR heterodimer is activated by its respective ligand.

The unavailability of radiolabelled LXR α activators prevents direct ligand-binding analysis at present. To investigate the possibility that 22(R)-HC could interact with LXR α , we used a limited protease-protection assay (Fig. 4) in which several proteolytic fragments were generated when LXR α protein was incubated with increasing concentrations of the protease chymotrypsin. Of these fragments, only a unique 30K fragment (arrow in Fig. 4, right panel) was consistently seen in the presence of 22(R)-HC but not ethanol (Fig. 4, left panel), 9-*cis* retinoic acid or cholesterol (data not shown). Specific ligand-protected fragments (~30K) have also been identified in similar analyses with other nuclear receptors¹⁹. These studies provide evidence that 22(R)-HC interacts with LXR α .

The ability of oxysterols to activate transcription through LXR α has implications for the function of these compounds *in vivo*. Their rank order of potency is distinct from that which modulates end-product repression of cholesterol through SREBP (M. S. Brown and J. L. Goldstein, personal communication), suggesting that these sterols have a new function as activators. For example, FF-MAS regulates meiosis⁶, implying LXR α or related receptors may function in the gonads. Further clues to the function of these

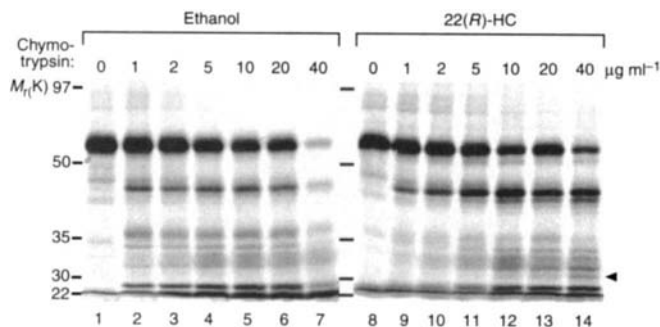


FIG. 4 Protease protection of LXR α with 22(R)-HC. ³⁵S-labelled LXR α protein was incubated with 10 μ M 22(R)-HC or ethanol control, digested with increasing amounts of chymotrypsin, and analysed by SDS-PAGE and autoradiography. Arrowhead depicts a novel digestion product specifically protected by 22(R)-HC.

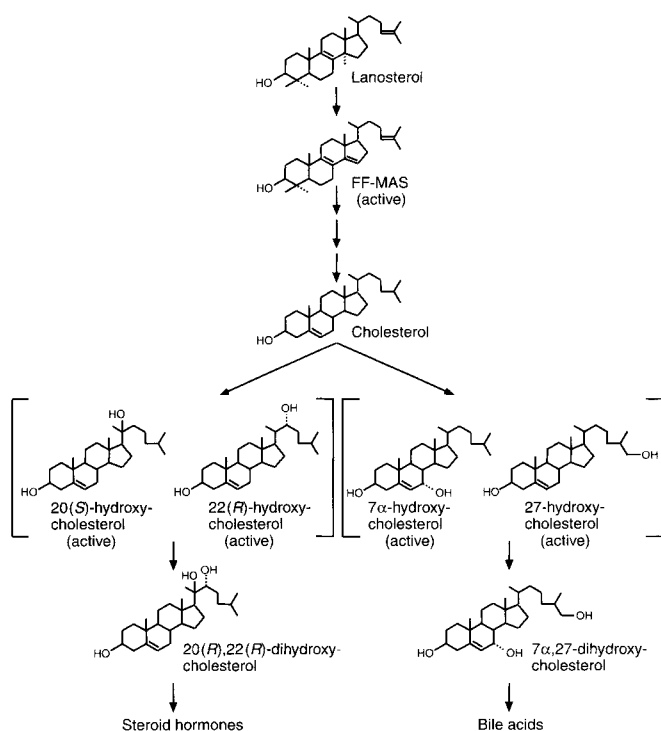


FIG. 5 Metabolic fates of oxysterols. LXR α activators FF-MAS, 20(S)-HC, 22(R)-HC, 7 α -HC and 27-HC are indicated and function at the rate-limiting steps of three important metabolic pathways: conversion of lanosterol to cholesterol, steroid hormone synthesis and bile acid synthesis.

sterols come from their metabolic fates (Fig. 5). LXR α activators exist at the rate-limiting steps of three important pathways: steroid hormone biosynthesis, bile acid synthesis, and conversion of lanosterol to cholesterol. Although further metabolism may yield more potent activators, our results show that the immediate upstream and downstream metabolites of these activators are significantly less potent (Figs 2a and 5). Furthermore, at least one of these activators, 22(R)-HC, requires the ligand-binding domain of LXR α for activation (Fig. 3c) and can protect the LXR α protein from limited proteolytic digestion (Fig. 4). Taken together, these results indicate that compounds such as 22(R)-HC may function as LXR α ligands. Alternatively, these oxysterols could function through a less direct mechanism. For example, the activators could interact with an LXR α -specific coactivator or corepressor protein similar to those that exist for other nuclear receptors^{20–22}. Radiolabelled binding studies should clarify the ligand-binding properties of these activators.

In many metabolic pathways, rate-limiting steps are often regulated by feedback or feedforward loops. We propose that LXR α may act as a sensor of specific sterols such as 22(R)-HC or 7 α -HC and thereby transcriptionally regulate a crucial metabolic pathway (for example, steroid hormone or bile acid biosynthesis). The pattern of LXR α expression is specific to tissues in which these pathways exist, such as liver, intestine and adrenals³. Our finding that LXR α mediates oxysterol-induced transactivation indicates that, as in the case of retinoids and steroids, a specific class of nuclear receptors may exist for oxysterols. □

Methods

Candidate ligands. Organic extraction of 12 g lyophilized breeding-bull testis was done as described⁶. Aliquots representing 1% of this material were assayed. FF-MAS was synthesized as described²³; 27-hydroxycholesterol was a gift from N. Javitt; 24-hydroxycholesterol was a gift from E. Lund and D. Russell; 7 α ,25-

dihydroxycholesterol was a gift from M. Schwarz and D. Russell; isomers of 20,22-dihydroxycholesterol were synthesized as described²⁴ or were donated by J. I. Mason. All other sterols and receptor ligands were purchased from Steraloids (Wilton, NH), Research Plus (Bayonne, NJ) or Sigma.

Transfections. Transient transfections in CV-1 monkey kidney cells were done as described³. Candidate ligands were dissolved in ethanol and added to cells after transfection in medium containing 5% cabosil-treated newborn-calf serum. Plasmids used were TK-MH100 $\times$ 4-LUC reporter and CMX-GAL4-hLXR α chimaeric receptor (Fig. 1a); TK-LXRE $\times$ 3-LUC reporter, CMX-hLXR α and CMX-hRXR α receptors (Figs 1b, 2, 3c, d)³. Expression plasmids for receptors¹⁷ and their cognate luciferase reporters used in Fig. 3a were: human LXR α , TK-LXRE $\times$ 3-LUC; human retinoid-X receptor- α (RXR α)²⁵, TK-CRBPII-LUC; human retinoic acid receptor- α (RAR α)²⁶, TK-DR5-LUC; human thyroid hormone receptor- β (TR β)²⁷, TK-DR4-LUC; human vitamin D receptor (VDR)²⁸, TK-DR3-LUC; human peroxisome proliferator-activated receptor- α (PPAR α)²⁹, TK-PPRE $\times$ 3-LUC; human farnesol-activated receptor (FXR)¹¹, Δ MTV-EcRE $\times$ 5-LUC; human oestrogen receptor (ER)³⁰, Δ MTV-ERE-LUC; human glucocorticoid receptor (GR)³¹, MTV-LUC; *Drosophila* ecdysone receptor (usp/EcR)¹⁶, Δ MTV-EcRE $\times$ 5-LUC. As a positive control for each receptor, cells were treated with saturating concentrations of their cognate ligands (data not shown). Chimaeric receptors used in Fig. 3c were constructed by fusing the cDNA encoding the human LXR α N terminus and DNA-binding domain (DBD) (amino acids 1–163) with the ligand-binding domain (LBD) of human TR β (amino acids 170–456) to make CMX-hLXR-TR, and by fusing the cDNA encoding the TR β N terminus and DBD (amino acids 1–169) with the LBD of LXR α (amino acids 164–447) to make CMX-hTR-LXR. In Fig. 3b, *Drosophila* SL-2 cells were transfected as described⁵ with A5C-hLXR α and A5C-hRXR α receptors, and ADH-LXRE $\times$ 2-LUC reporter plasmids. β -Galactosidase expression was included in all transfections as an internal standard. Data are presented as mean relative light units (RLUs) from triplicate assays $\pm$ s.e., or as the fold induction of 22(R)-HC-induced activation over ethanol controls.

Protease-protection assay. *In vitro* synthesized ³⁵S-labelled Flag-LXR α protein was digested with α -chymotrypsin¹⁹. LXR α with a Flag epitope fused to the N terminus was used (the Flag epitope increases the efficiency of translation and does not interfere with LXR α activity as determined by DNA binding and transfection studies).

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Targeted ribose methylation of RNA *in vivo* directed by tailored antisense RNA guides

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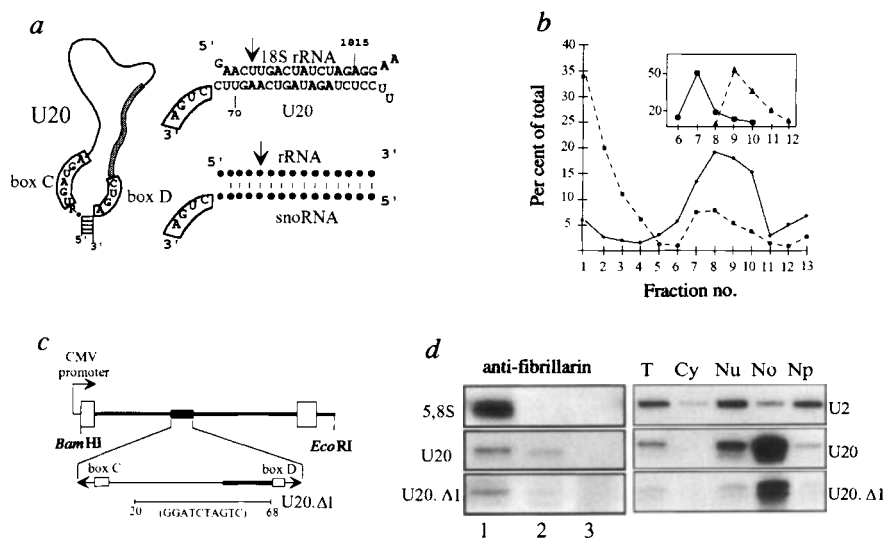
EUKARYOTIC ribosomal RNAs are post-transcriptionally modified by methylation at the ribose sugar of specific nucleotides¹. This takes place in the nucleolus and involves a family of small nucleolar RNAs (snoRNAs) with long regions (10–21 nucleotides) complementary to rRNA sequences spanning the methylation site^{2–4}—a complementary snoRNA is required for methylation at a specific site⁵. Here we show that altering the sequence of the snoRNA is sufficient to change the specificity of methylation. Mammalian cells transfected with a snoRNA engineered to be complementary to an arbitrary rRNA sequence direct the methylation of the predicted nucleotide in that sequence. We have further identified structural features, both of the guide and substrate RNA, required for methylation and have used these to design an exogenous transcript, devoid of rRNA sequence, that is site-specifically methylated when co-expressed with an appropriate guide snoRNA. Endogenous non-ribosomal RNA can thus be targeted, possibly providing a highly selective tool for the alteration of gene expression at the post-transcriptional level.

The small nucleolar RNA U20 (ref. 6) belongs to a growing family of fibrillarin-associated intronic snoRNAs with long, conserved complementarities to rRNA^{2,3} which contain the box C and box D motifs (RUGAUGA and CUGA, respectively) brought close together through pairing of the 5' and 3' terminal nucleotides (Fig. 1a). The rRNA complementarities of these snoRNAs span sites of 2'-O-ribose methylation in rRNA². Remarkably, in snoRNA/rRNA duplexes the modified site in rRNA is always

paired to the fifth nucleotide upstream from box D, strongly suggesting that snoRNA pairing provides the methyltransferase with the appropriate site specificity on pre-rRNA^{4,5}. Ribose methylation of rRNA takes place either on the elongating transcript or on newly formed 45S pre-rRNA^{1,7}. To detect base pairing between U20 and nascent pre-rRNA, we analysed deproteinized nuclear RNA in non-denaturing conditions^{8,9}. In exponentially growing cells, about 85% of U20 was broadly distributed in the 28S–45S range (Fig. 1b), consistent with its association with elongating transcripts carrying the snoRNA complementarity which is located 5 kilobases (kb) from the transcription start site, near the 3' end of 18S rRNA⁶. In agreement with this, the amount of U20 co-sedimenting with pre-rRNAs dramatically decreased (Fig. 1b) shortly after addition to the cell culture of a low dose of actinomycin D, which selectively blocks rRNA transcription¹⁰, when the nuclear content of preformed rRNA precursors has not yet substantially decreased (data not shown). Exogenous U20 can be transiently expressed in mouse cells transfected with constructs in which the U20-containing intron and the two flanking exons of the nucleolin gene are transcribed under the control of the CMV promoter (Fig. 1c). Accumulation of a stable, faithfully processed U20 is independent of the rest of the intron sequence and is essentially directed by the nucleotides forming the 5'–3' terminal stem-box structure in the snoRNA^{11,12}. Thus, deletion of most of the snoRNA coding sequence between box C and box D did not prevent the formation of a correctly processed small RNA which was targeted to the nucleolus at levels very similar to endogenous U20, and was still immunoprecipitated by anti-fibrillarin antibody at about 50% of the control level (Fig. 1d).

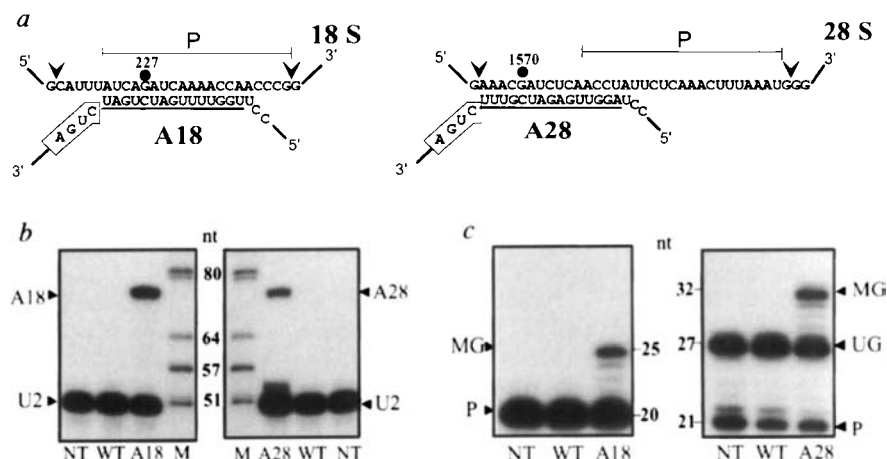
To test whether formation of a snoRNA/rRNA canonical duplex^{4,5} would be sufficient to specify a site of ribose methylation in endogenous rRNA, we studied U20 mutants in which the natural 18S rRNA complementarity had been substituted by a complementarity to another rRNA sequence, naturally devoid of sugar-methylated nucleotide, in 18S or in 28S rRNA (Fig. 2a). Each of the two 16-nucleotide artificial antisense elements was chosen to form a duplex in which the targeted rRNA nucleotide, paired to the fifth position upstream from box D in the snoRNA^{4,5}, was a guanosine. Given that the phosphodiester bond immediately

FIG. 1 Direct association of antisense snoRNA U20 with nascent pre-rRNA and expression of U20 mutants. **a**, The snoRNA/rRNA complementarity. Left, schematized structure of U20 snoRNA^{2,6} (thick line: 18S rRNA complementarity). Right, conserved base pairings between U20 and 18S rRNA (top) and the canonical duplex^{4,5} (bottom) between the family of U20-related snoRNAs and rRNA sequences around a site of 2'-O-ribose methylation (arrow). **b**, Distribution of endogenous U20 on non-denaturing sucrose gradients. Nuclear RNA from exponentially growing HeLa cells collected either directly or 5 min after addition of 0.1 $\mu\text{g ml}^{-1}$ actinomycin D¹⁰ (continuous and broken lines, respectively) was isolated under non-denaturing conditions^{8,9}. The U20 content of gradient fractions was assayed by northern blotting. Inset: distribution of 32S and 45S pre-rRNAs (continuous and broken lines, respectively) in untreated cells (northern with a 5.8S rRNA probe after electrophoresis on a 0.9% agarose/formaldehyde gel). **c**, Construct expressing U20 mutants in transfected mouse cells. The fragment of the mouse nucleolin gene²⁷ spanning the U20 (filled box)-containing intron and flanking exons (open boxes). In mutant $\Delta 1$, segment 20–68 is replaced by a 10-nt foreign sequence (in parentheses). **d**, Interactin with fibrillarin and nucleolarization of mutant U20. $\Delta 1$. Left, immunoprecipitation with anti-fibrillarin antibody²⁸ was done on a transfected cell extract prepared in 150 mM NaCl. Lanes: 1, RNA from an aliquot



of extract corresponding to 10^6 cells; 2 and 3, RNA precipitated by antifibrillarin or a non-immune serum, respectively, from an aliquot of the cell extract corresponding to 10^7 cells. Right, RNAs were purified²⁹ from total transfected cells (T) and from cytoplasmic (Cy), nuclear (Nu), nucleolar (No) and nucleoplasmic (Np) fractions. Mutant U20. $\Delta 1$ RNA was assayed by reverse transcription with a specific primer and wild-type U20 by northern blotting; U2 snRNA and 5.8S rRNA were also assayed by northern blot.

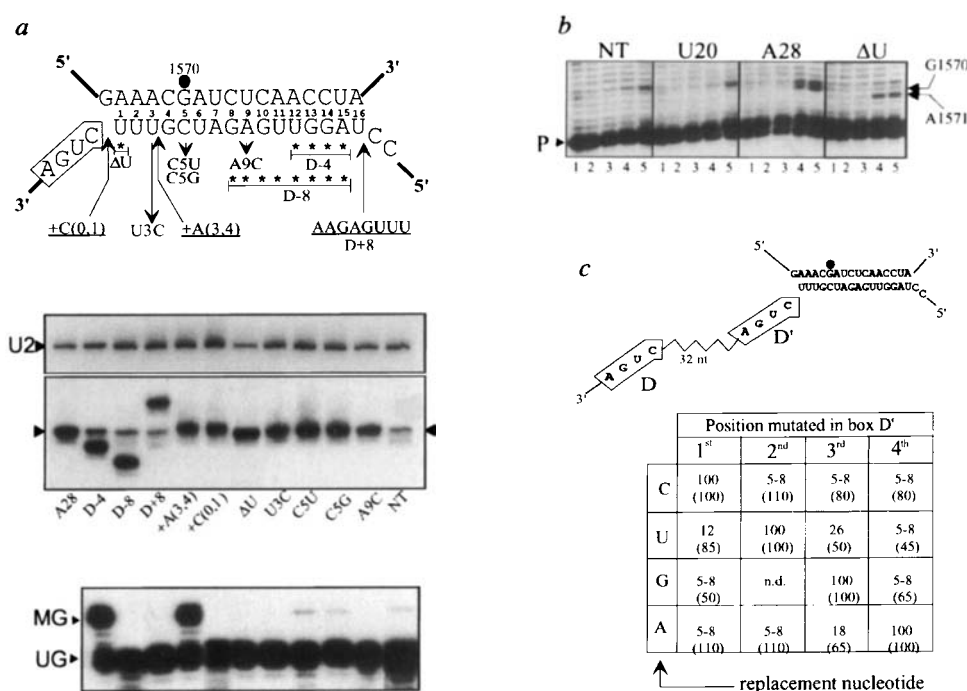
FIG. 2 Targeted 2'-O-ribose methylation of 18S and 28S rRNA directed by tailored U20 snoRNA mutants. **a**, Duplexes formed by mutants A18 and A28 at the rRNA target sites (mouse coordinates). In the mutants the artificial antisense element (underlined) replaces segment 56–70 of the mouse U20 sequence⁶. The guanosine target is denoted by a filled circle. Arrowheads: termini of the RNase T1 oligoribonucleotide produced when the target was ribose-methylated; P, oligodeoxynucleotides used for primer extension analysis of the RNase T1 digest. **b**, Expression of U20 mutants with artificial antisense elements. Total RNA from transfected cells was assayed by reverse transcription with A18- and A28-specific primers (a U2 snRNA-specific primer provided an internal control for gel loading). RNAs from non-transfected cells (NT) and from cells transfected with a construct expressing wild-type U20 (WT) were analysed in parallel. nt, Number of nucleotides. **c**, Detection of novel 2'-O-ribose methylations in 18S rRNA (left) and in 28S rRNA (right). Primers shown in **a** were extended by reverse transcription of an RNase T1 digest of total cellular RNA. The cDNA band termed MG reflects ribose-methylation of the guanosine target.



downstream from a 2'-O-ribose methylated guanosine¹ is resistant to ribonuclease (RNase) T1, ribose methylation of a guanosine far enough from the next downstream G can be assayed by primer extension (Fig. 2a). We found that transfected cells accumulated the two artificial forms of U20 RNA, A18 and A28, to about the same extent (Fig. 2b), roughly comparable to the level of endogenous U20 (Fig. 3a). After complete digestion of total cellular RNA by RNase T1, oligoribonucleotides bearing the T1-resistant targeted guanosine were detected using primer extension (MG bands in Fig. 2c). For a non-methylated G1570 in 28S rRNA, a

shorter cDNA (UG band) is produced from the same primer, thus allowing the extent of modification at this site to be evaluated. Considered with the low turnover of mature rRNA produced before transfection, the relatively high value that was derived, 30–40%, indicates that a major part of the rRNA molecules synthesized after expression of A28 had undergone the targeted modification. We found that the relative intensities of the two bands remained similar when the assay was done on 28S rRNA extracted from cytoplasmic 60S ribosomal subunits¹³ instead of on total cellular RNA, or after a 9-hour block of transcription by 5 $\mu\text{g ml}^{-1}$

FIG. 3 Identification of the snoRNA nucleotides involved in methylation guide function. **a**, Effects of alterations of the canonical duplex structure on the level of methylation. Top: mutations introduced in the A28 derivative, with single substitution mutants termed according to the position mutated in the duplex and mutants resulting in a modified duplex length termed D – 4, D – 8 and D + 8 (stars, deletions; underline nucleotides, insertions). Middle: accumulation of guide RNAs in transfected cells, analysed by northern blotting with a probe recognizing both endogenous U20 (arrowhead) and mutant U20. Abundance of mutants co-migrating with wild-type U20 was estimated by subtracting the endogenous U20 signal, using U2 snRNA as an internal reference. Bottom: ribose methylation at G1570 of 28S rRNA in transfected cells assayed by primer extension on total cellular RNA digested by RNase T1, as in Fig. 2c (the MG-to-UG band signal ratio reflected the extent of reaction). **b**, Shift of the methylation site provoked by the ΔU mutation detected by reverse transcription with decreasing concentrations of dNTPs (1 mM, 200 μM , 40 μM , 4 μM and 1 μM in lanes 1–5, respectively), owing to concentration-dependent pauses at ribose-methylated sites in rRNA³⁰. NT, non-transfected cells; U20 and A28, transfection with wild-type U20 or A28. **c**, Effect of alterations of the CUGA motif. Top, the 16-nucleotide (nt) antisense element of A28, linked to a downstream CUGA (box D'), was inserted 32 nt upstream from the natural box D of a U20 derivative and



single nucleotide changes introduced in the additional CUGA. The table shows the amount of methylation at G1570 in 28S rRNA with mutants carrying the indicated change in the CUGA, relative to the same snoRNA with an intact CUGA (cellular abundance of the mutants relative to the same reference in brackets).

actinomycin D (not shown), showing that novel ribose methylation at G1570 did not inhibit production of mature 28S rRNA or alter its stability.

Elements of snoRNA structure essential for site-specific methylation were tested by assaying the extent of modification in A28 mutants (Fig. 3a). All mutants accumulated to roughly similar levels in transfected cells, about 2–3 times higher than endogenous U20 in most cases. Extending the rRNA complementarity upstream from box D to 24 base pairs (bp) did not affect the level of ribose methylation at G1570, but shortening it to 7 or even to 12 bp completely abolished it. Natural antisense snoRNAs associated with ribose methylation in rRNA may form duplexes as short as 10 base pairs⁴, suggesting that the minimal length of a functional complementarity may vary according to the rRNA site and perhaps the thermodynamic stability of the duplex and local conformation of the pre-ribosomal substrate. Most single mutations creating non-Watson–Crick base appositions in the core of the 16-bp duplex also blocked the reaction. Thus, methylation of G1570 was completely abolished by placing a G in opposite position (C5G mutation). However, there was a low but significant level of reaction (~1% of control) for a less destabilizing, non-canonical G–U base pair at the target site (C5U mutation) and for two other non-canonical base appositions in the duplex (U3C and A9C mutations). Insertions or deletions within the antisense element completely suppressed ribose methylation at G1570, as shown for mutants C(0,1), A(3,4) and Δ U. In mutant Δ U, deletion of the nucleotide upstream from box D shortens the antisense element by one nucleotide, modifying its spacing relative to the CUGA. In this case, the methylation was shifted (Fig. 3b), as expected, to A1571, the position in the duplex paired to the fifth nucleotide upstream from box D^{4,5}. As biosynthesis of a stable intron-encoded, fibrillar-associated snoRNA depends on an

intact box D^{11,12,14}, the importance of this motif for methylation guide function could not be tested with A28. We used derivatives with a second CUGA, similar to a subset of natural snoRNAs which have a more upstream antisense element, followed immediately by another copy of box D^{4,5,15}. Insertion, much further upstream from the natural box D of U20, of the 16-bp antisense element of A28 followed by an additional CUGA (Fig. 3c) preserved both the production of a stable snoRNA and the site-specific methylation of G1570 in 28S rRNA (not shown). Single mutations in the additional CUGA did not dramatically alter snoRNA accumulation but drastically affected ribose methylation of the target (Fig. 3c). As box D' in several natural antisense snoRNAs shows a one-nucleotide deviation from the CUGA^{4,5,15}, this suggests that other features of the snoRNP structure might contribute to its activity.

We next examined whether, in addition to the rRNA nucleotides in the duplex, other features of the rRNA molecule were required for site-specific ribose methylation *in vivo*. We selected as exogenous substrate a 20-nucleotide-long 28S rRNA segment (Fig. 4a) bearing a guanosine, which is naturally ribose-methylated in endogenous rRNA, together with all the nucleotides likely to participate in the canonical duplex with the cognate endogenous guide snoRNA, snR39B (ref. 4). To direct its nucleolarization, this substrate was transcribed by RNA polymerase I from a rRNA minigene¹⁶. In the minigene transcript, which was devoid of any other mature rRNA sequence, the 20-nucleotide 28S rRNA segment was inserted downstream of a long G-lacking sequence, again to facilitate detection of ribose methylation at the target site by RNase T1 digestion (Fig. 4a). In the minigene transcript, which localized preferentially to the nucleolus, the target guanosine was ribose-methylated at more than 90%, very similar to the endogenous substrate (Fig. 4a). As expected, the modification was

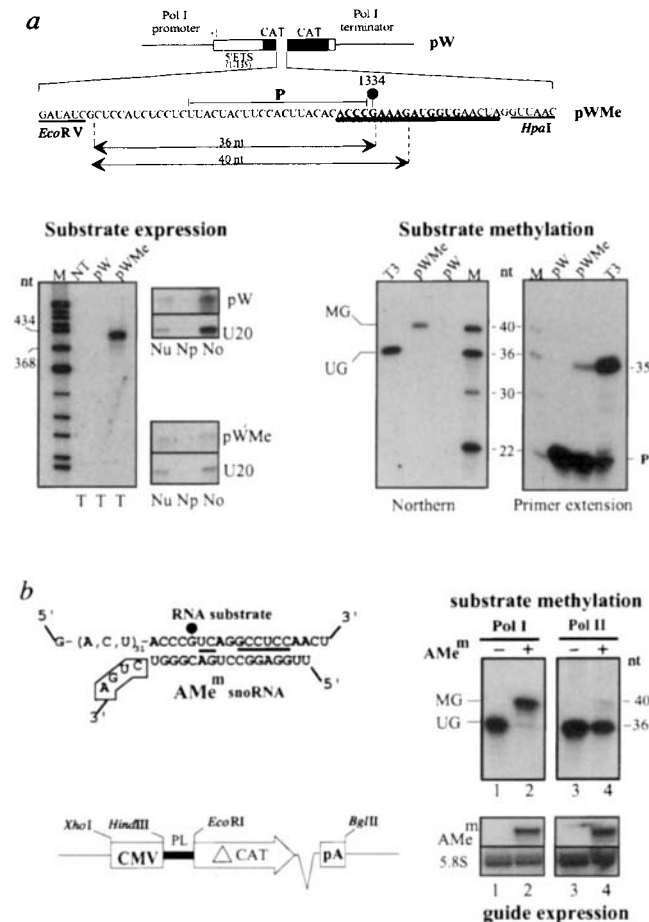


FIG. 4 Site-specific ribose methylation of exogenous RNA sequences *in vivo*. **a**, Methylation of an RNA polymerase I transcript (top panel) driven by an endogenous snoRNA guide. Mouse rRNA minigene pW (ref. 16) contains the RNA polymerase I promoter and terminator, terminal nucleotides of the 5' and 3' ETS (open boxes) and a chloramphenicol acetyltransferase (CAT) gene fragment. Minigene pWMe was derived by inserting into the polylinker of pW the DNA fragment coding for the depicted 65-nt RNA sequence containing a 20-nt tract (underlined) of 28S rRNA (in endogenous rRNA nucleotides in bold pair with snR39B⁴, which guides the ribose methylation of G1334 (filled circle); RNase T1 oligoribonucleotides reflecting the presence or absence of ribose methylation at G1334 are shown. P, probe and primer). Bottom left, expression and intranuclear localization of the minigene transcript. Total RNAs (lanes T) from non-transfected mouse cells (NT) or from cells transfected with pWMe or pW were analysed by northern blotting with probe P. RNA aliquots extracted from isolated nuclei (Nu) and from nucleolar (No) and nucleoplasmic (Np) fractions of cells transfected with pW or pWMe were assayed with a minigene-specific probe. Bottom right, ribose methylation at G1334 in the minigene transcript. Total cellular RNA (30 μ g) digested by RNase T1 was analysed by northern (left) with probe P. To provide for an unmethylated control fragment (band UG, lane T3), the same insert was transcribed *in vitro* by T3 RNA polymerase and the transcript also digested by RNase T1. Identity of the 40-nt band (MG) was confirmed by primer extension (right) of probe P, using the same RNase T1 digests. **b**, Reaction driven by a tailored exogenous guide onto a non-ribosomal RNA sequence transcribed by RNA polymerase I or II. Left, structure of the canonical guide duplex between the exogenous substrate and the U20 snoRNA derivative AME^m. The RNA polymerase I transcript substrate was as in **a**, except for mutations (underlined) in the 20-nt 28S rRNA tract. The RNA polymerase II transcript contained the same 65-nt RNA fragment with the same mutations. The construct was obtained by inserting the 65-bp DNA fragment in the polylinker (PL) of the vector schematized below (P. Bouvet, unpublished results), derived by insertion into the polylinker of pSP72 (Promega) of the cytomegalovirus (CMV) promoter, a CAT gene fragment and the SV40 intron and polyadenylation signal. Right, ribose methylation of the targeted guanosine was assayed as in **a**, in cells transfected solely by the construct expressing each reaction substrate (lanes 1 and 3) and in cells cotransfected with the guide-expressing construct (lanes 2 and 4). Accumulation of AME^m snoRNA was analysed by northern blot with a specific probe on 5 μ g of total cellular RNA (endogenous 5.8S RNA served as an internal reference).

abolished by mutations in the 20-nucleotide rRNA sequence preventing formation of the canonical duplex with the endogenous guide snoRNA (Fig. 4b, lane 1). However, the targeted reaction was very efficiently restored by co-expression of a tailored snoRNA, AMe^m, with compensatory base changes in its antisense element (Fig. 4b, lane 2). Remarkably, when the insert bearing the mutated 20-nucleotide rRNA sequence was transcribed by RNA polymerase II instead of RNA polymerase I, co-expression of the same cognate snoRNA guide resulted in a low but still significant level of methylation (~10%) of the exogenous substrate (Fig. 4b, lane 4), suggesting that nucleolar localization of the RNA substrate is important but not essential for the reaction. In conclusion, the pairing of a family of antisense snoRNAs, which could also have a chaperone function in the folding of pre-rRNA^{2,15,17}, is sufficient to direct site-specific ribose methylation of rRNA. Guide activity of the snoRNA is encoded in its antisense element and the vicinal CUGA, a motif that specifies the nucleotide to be modified in the duplex, in addition to being involved in faithful processing^{11,12} and nucleolar targeting of intronic snoRNAs. This provides a framework for studying the ribose methylation machinery, particularly the methyltransferase, and the function of those rRNA modifications that are not essential for pre-rRNA processing^{18,19} but are likely to be important in mature ribosomes¹. Clearly, the potential for site-directed methylation *in vivo* is not restricted to rRNAs, although nucleolar localization of the substrate seems to be important for efficient reaction. Given the role of the nucleolus in messenger RNA transport^{20,21}, targeted ribose methylations could be used to alter the processing of mRNA^{22,23}, or modulate its function, possibly in a tissue-specific manner, using appropriate host genes for artificial intronic guide snoRNAs. Finally, ribose methylation of rRNA represents another post-transcriptional modification of RNA directed by duplexes involving intronic RNA segments²⁴, supporting the idea that intron-encoded genetic information mediated by RNA may contribute to a variety of regulatory mechanisms. □

Methods

Mutagenesis, transfection and snoRNA expression. After oligodeoxynucleotide-directed site-specific mutagenesis²⁵ of the U20 sequence, mouse L929 cells were transfected¹⁶ by the DEAE-dextran method and expression of snoRNAs assayed after 48 h by northern blotting (electrophoresis on 6 or 8% acrylamide/7 M urea gels) with appropriate oligonucleotide probes (cells were co-transfected with equal concentrations of the two plasmid DNAs). Intensities of radioactive bands were measured on a Phosphorimager.

RNA analysis in non-denaturing conditions. Nuclei were isolated and treated by DNase (ref. 8). After addition of proteinase K in the presence of LDS, the lysate was centrifuged on a 10–30% sucrose gradient⁹. The U20 content of gradient fractions was assayed by northern blotting with a 5' ³²P-labelled probe complementary to segment 8–27 of the mouse U20 sequence⁶.

Digestion with RNase T1. After two ethanol precipitations in the presence of 0.8 M LiCl to eliminate low-molecular-weight RNAs, cellular RNA was completely digested by RNase T1 (ref. 26). After the first treatment (with 600 units for 30 min at 37 °C; 20 µg aliquots in 10 µl) followed by phenol extraction and ethanol precipitation, RNA was heat-denatured (2 min at 100 °C) and quickly chilled at 0 °C before a second incubation with RNase T1 (1,800 units for 1 h at 65 °C).

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A new class of uracil-DNA glycosylases related to human thymine-DNA glycosylase

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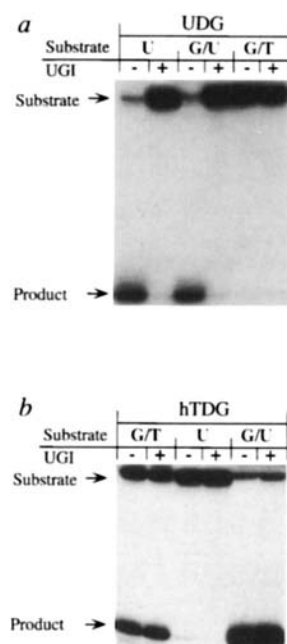
MISPAIRS in DNA of guanine with uracil and thymine can arise as a result of deamination of cytosine and 5-methylcytosine, respectively. In humans such mispairs are removed by thymine-DNA glycosylase (TDG)^{1–3}. By deleting the carboxy and amino termini of this enzyme we have identified a core region capable of processing G/U but not G/T mispairs. We have further identified two bacterial proteins with strong sequence homology to this core and shown that the homologue from *Escherichia coli* (dsUDG) can remove uracil from G/U mispairs. This enzyme is likely to act as a back-up to the highly efficient and abundant enzyme uracil-DNA glycosylase (UDG) which is found in most organisms. Pupating insects have been reported to lack UDG activity⁴, but we have identified an enzyme similar to dsUDG in cell lines from three different insect species. These data imply the existence of a family of double-strand-specific uracil-DNA glycosylases which, although they are subservient to UDG in most organisms, may constitute the first line of defence against the mutagenic effects of cytosine deamination in insects.

Uracil or thymine glycosylase activities were assayed by incubating synthetic oligonucleotides containing a single uracil residue or duplexes containing G/U or G/T mispairs with cell extracts, *in vitro* translation mixtures (reticulocyte lysates) or with purified recombinant proteins as described previously^{1–3}. As shown in Fig. 1a, the *Escherichia coli* UDG enzyme could process both U and G/U, but not G/T, substrates. In contrast, the purified human TDG enzyme⁵ failed to process the single-stranded uracil-containing substrate, but could efficiently remove both thymine and uracil from duplex substrates in which these bases were mispaired with guanine³ (Fig. 1b). The two uracil glycosylases could be readily distinguished from each other, because the former enzyme was inhibited by UGI peptide (a PBS2 phage-encoded competitive inhibitor of UDG)⁶ (Fig. 1a, lanes +UGI), whereas the TDG activity was insensitive to the presence of UGI (Fig. 1b, lanes +UGI).

We expressed a series of N- and C-terminal hTDG-deletion mutants in a cell-free reticulocyte lysate system and the crude reaction mixtures were used in enzyme assays on G/U and G/T substrates. Figure 2a shows that deletion of the N-terminal 56 amino acids of TDG yielded an enzyme (5'Δ56) active on both substrates, whereas the loss of a further 56 amino acids abolished

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FIG. 1 Processing of single- or double-stranded 34-mer oligonucleotide substrates containing uracil (U) or G/T or G/U mispairs¹ by the *E. coli* uracil DNA-glycosylase (UDG) and the human thymine DNA-glycosylase (hTDG)⁵. a, Substrates were incubated with purified UDG in the presence (+) or absence (-) of UGI inhibitor. b, U, G/U or G/T substrates were incubated with affinity-purified hTDG; an autoradiogram of a 20% denaturing polyacrylamide gel is shown.

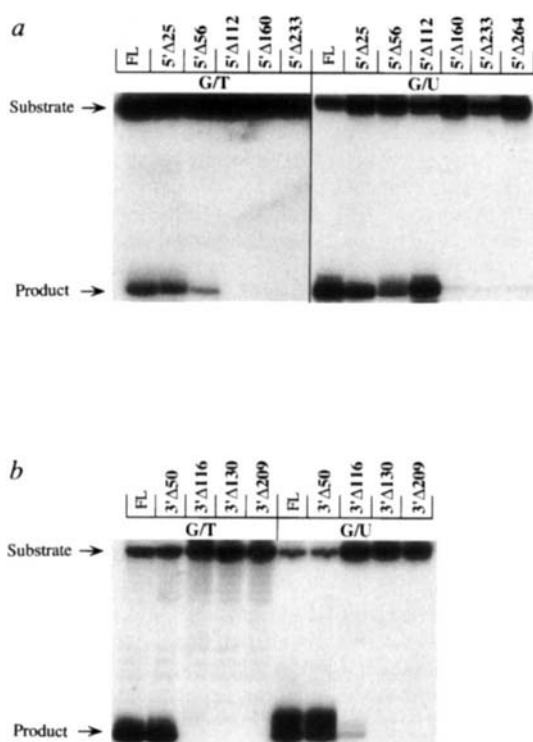


activity on G/T but not G/U (5'Δ112). The mutant 5'Δ112 appears to be more active than the full-length protein on the G/U substrate, an observation that must be verified using purified protein; our preliminary results indicate that the 112-amino-acid deletion leads to an increased turnover of the enzyme. Thus, although TDG and its deletion mutants 5'Δ25 and 5'Δ56 form apparently stable complexes with the substrate oligonucleotide duplexes (as measured by gel-shift assays), the 5'Δ112 mutant does not (data not shown). Deletions of more than 112 amino acids from the N terminus and C-terminal deletions of more than 50 amino acids (Fig. 2b) lead to a more than 20-fold decrease of both activities, although the mutant 3'Δ116 appears to have above-background residual activity (Fig. 2b).

These data indicate that only the central 249 amino acid

residues of the enzyme appear to be necessary for G/U recognition and for uracil glycosylase activity, whereas G/T processing requires additional amino acids at the N terminus of the polypeptide (Fig. 2c)—this latter finding suggests that the extra N-terminal domain has a possible role in G/T processing. The crystal structures of human⁷ and herpes simplex virus⁸ uracil glycosylases showed that the active sites of these enzymes are extremely tight-fitting, excluding thymine as a possible substrate. Although TDG does not appear to be related to UDG, the 5'Δ112 TDG deletion mutant may have similar exclusion criteria. The TDG amino terminus may alter the structure of the active site so that it can also accept thymine within the context of a G/T mispair: on-going structural studies should provide an answer.

Although amino-acid sequence analysis of hTDG failed to identify peptide motifs conserved in other enzymes involved in DNA repair or metabolism⁹, databank searches revealed a surprising degree of homology between the amino-acid sequence of hTDG and those of two bacterial open reading frames (ORFs). Interestingly, the conservation is limited to the central part of hTDG, which encodes the G/U-specific uracil glycosylase (Fig. 3). We expressed the 169-amino-acid *E. coli* ORF to see whether the encoded protein had uracil glycosylase activity. As anticipated, the recombinant bacterial polypeptide, dsUDG, expressed either in reticulocyte lysates (Fig. 4a) or in *E. coli* (data not shown), could efficiently process G/U but not G/T mispairs. As this enzyme was not inhibited by UGI (data not shown), the protein must be distinct from UDG. The minimal active domain of TDG (5'Δ112/3'Δ50) expressed in the same system had similar activity on the substrates tested (Fig. 4a). We tested for dsUDG activity *in vivo* by examining extracts of two isogenic *E. coli* strains, NR8051 and NR8052; the latter carries the *ungI* mutation¹⁰ and has a significantly reduced level of UDG activity¹¹. Both U and G/U were processed in the UDG⁺ NR8051 extract and cleavage of the former was inhibited with UGI (Fig. 4b); in contrast, only the G/U oligonucleotide was efficiently processed in the NR8052 (UDG⁻) extracts. The enzyme responsible for this activity was insensitive to UGI, indicating that processing was not mediated by UDG. Evidence that the double-strand-specific uracil DNA-glycosylase in *E. coli* extracts (Fig. 4b) is encoded by the dsUDG locus comes



c		Enzymatic activity on	
		G/T	G/U
FL	I	+	+
5' Δ25		+	+
5' Δ56		+	+
5' Δ112		-	+
5' Δ160		-	-
5' Δ233		-	-
5' Δ264		NT	-
3' Δ50		+	+
3' Δ116		-	-
3' Δ130		-	-
3' Δ209		-	-

112 ——— minimal functional domain on G/U ——— 360
56 ——— minimal functional domain on G/T ——— 360

FIG. 2 Deletion mutagenesis of hTDG. Nicking assays of *in vitro* translated deletion mutants with G/T and G/U 34-mer substrates are shown. a, Nicking activity of full-length hTDG (FL) and of peptides with N-terminal deletions of 25, 56, 112, 160, 233 and 264 amino acids (lanes 5'Δ25–264, respectively). Enzyme activity of 5'Δ264 on G/T was not tested. b, Nicking activity of full-length hTDG (FL) and of peptides with C-terminal deletions of 50, 116, 130 and 209 amino acids (lanes 3'Δ50–209); a, b show autoradiograms of 20% denaturing polyacrylamide gels. c, Deletion mutants and their respective activities on G/T and G/U substrates. +, Enzyme activity >5% of that observed with full-length hTDG. The minimal functional domains are shown as black bars. Numbers represent amino-acid residues. NT, not tested.

Human	MEANAGSYSLQQAQAFYTFPFQQLMAEAP	30
Mouse	MDAEARSYSLEQVQALYSFPFQQMMAEVP	30
	5' Δ 112	
Human	NMAVVNEQQMP.....EEVPAPAPAEQEPVQEAPEKGRKRKPRTPTEPKQVPEPKPVESKSGKSAKPKEKQEKITDTPKVKRKVDKRVNGVSEAE	119
Mouse	NMAVTTGQQVPAPVAPNMTATVEQQVPADAPVQEPAPAEKRRKRKPRAAEPQEPVPEKKPATSKSGKSTKSKEKQEKITDAFKVKRKVDKRVNGVSEAE	130
	5' Δ 160	
Human	LTKTLPDILTFNLDIVIIIGINPGLMAAYKGHHYPGPGNHFWKCLFMSGLSEVQLNHMDHTLPKGYGIGFTNMVERTTPGSKDLSSEKPFREGGRILVQKL	219
Mouse	LTKTLPDILTFNLDIVIIIGINPGLMAAYKGHHYPGPGNHFWKCLFMSGLSEVQLNHMDHTLPKGYGIGFTNMVERTTPGSKDLSSEKPFREGGRILVQKL	230
E. coli	...MVEDILAPGLRVVFCGINPGLSSAGTGFFFAHPANRFWKVIYQAGFTDRQLKPQEAQHLL.DYRCGVTKLVDRPTVQANEVSKQELHAGGRKLEKI	96
S. marcescens	MELLAPNLRVVFCEINPGLSSAHQGYPPFANGSNRFWKVIHQAGFTESQLAPEQWQQLK.DNCGGITALVARPTVAASELSRDELRSAGEALQEKI	94
	3' Δ 116	
Human	QKYQPRIAVFNGKCIYEIIFSKEVFGVKVKNLEFGLQPHKIPDTETLCYVMPSSSARCAQFPRAQDKVHYIYIKLKDLDQLKGIERNMDVQEVQYTFDLQL	319
Mouse	QKYQPRIAVFNGKCIYEIIFSKEVFGVKVKNLEFGLQPHKIPDTETLCYVMPSSSARCAQFPRAQDKVHYIYIKLKDLDQLKGIERNMDVQEVQYTFDLQL	330
E. coli	EDYQPALATLGGQAYEQ.....GFSQGAQWKGKQTLTIGSTQIWLPLNPSGLSR.....VSLEKLVAYRELDQALVVRGR	168
S. marcescens	LRYPRALATLGGQAYFTT.....AFGVKNAPWGKQTLTIGETEVWVLPNPSGLNR.....ATLEQLTASYRELFLALQ	164
	3' Δ 50	
Human	AQEDAKKMAVKEEKYDPGYEAAAYGGAYGENPCSSPCGFSNGLI.ESVELRGESAFSGIPNGQWMTQSPTDQIPFSFNHCGTQEEESHA	410
Mouse	AQEDAKKMAVKEEKYDPGYEAAAYGGAYGENPCGNEPCGIASNGLTAHSAEPRGEATPGDVNPGQWMAQSFAEQIPSF.NNCGTREQEESHA	421

FIG. 3 Sequence alignment of the human and mouse TDG with the *E. coli* and *S. marcescens* ORFs encoding the double-stranded UDG activities. Conserved residues are in shaded boxes. Filled triangles indicate the last

active hTDG deletions, empty triangles the first inactive mutants. For sequence data, see Methods.

from a northern blot, in which we detected a unique band ~700 nucleotides that hybridized with the dsUDG complementary DNA probe (Fig. 4c). This indicates that the locus is transcribed into a monocistronic messenger RNA, because its length is only slightly greater than the 507-nucleotide ORF of dsUDG (Fig. 3). Evidence for this monocistronic dsUDG transcript also comes from analysis of the genomic locus, which has both consensus transcription initiation and termination signals (P. Visca, P.G. and J.J., unpublished results).

Although UDG was believed to be ubiquitous, the removal of uracil from the DNA of the fruitfly *Drosophila melanogaster* has been called into question. Thus, one group failed to find uracil glycosylase in *Drosophila* but described a developmentally regulated uracil-specific DNA endonuclease that appeared exclusively in third instar larvae⁴, proposing that this activity functioned in apoptosis-associated degradation of DNA during metamorphosis¹². In contrast, others detected uracil DNA-glycosylase both in egg and third instar larvae extracts that acted preferentially on supercoiled plasmid substrates containing G/U mismatches¹³. We have re-examined the issue with extracts of three pupating insect cell lines. We detected a small amount of nicking of the U substrate (Fig. 5) in extracts of the late embryo-derived *D. melanogaster* S2 cell line (Schneider's line 2; ref. 14). Although weak, the single-strand processing activity was inhibited by the UGI peptide, which suggests that it was due to a UDG-like homologue. Interestingly, this activity was completely absent from the extracts of the other two lines, Sf9 (derived from the pupa of the fall armyworm *Spodoptera frugiperda*) and H5 (derived from an early embryo of the cabbage looper *Trichoplusia ni*). In contrast, all three extracts contained significant amounts of a G/U processing activity which was not due to a UDG-like uracil glycosylase as the reaction could not be inhibited by UGI (Fig. 5, lanes +UGI). We showed that the enzyme in the insect extracts released uracil from the mispair (data not shown), by using a G/U substrate carrying ³H-uracil. It is thus a *bona fide* G/U-specific uracil-DNA glycosylase, probably a TDG homologue.

The presence of dsUDG in the insect extracts supports the hypothesis that all organisms must counter the mutagenic threat of cytosine deamination as the inability to repair G/U mispairs is incompatible with the long-term survival of the species¹⁶. But is there a need for two enzymes? During periods of rapid proliferation, when large amounts of uracil may be incorporated into DNA,

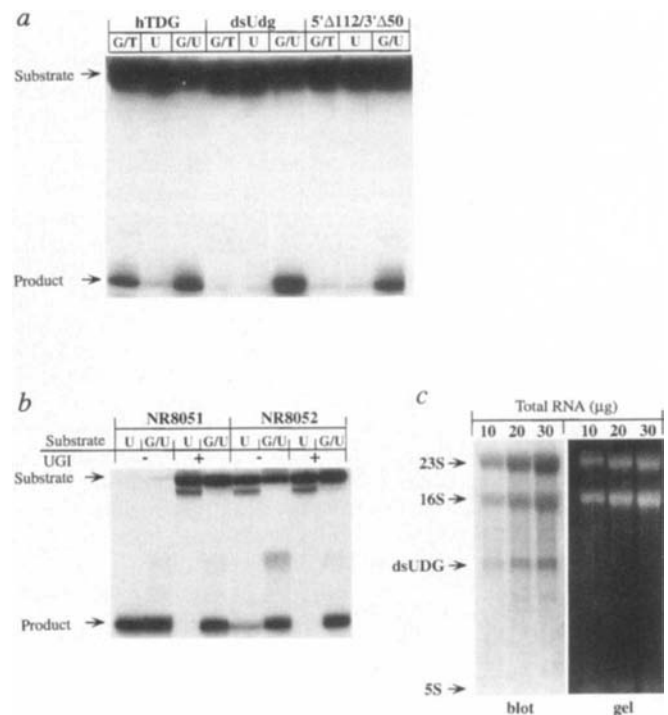
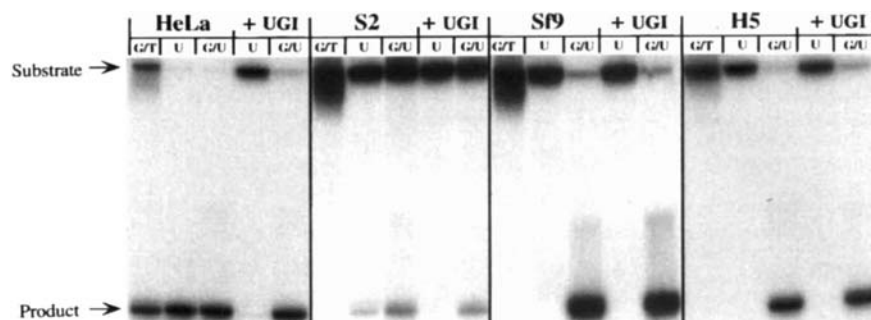


FIG. 4 a, Processing of uracil-containing single- (U) and double-stranded (G/T and G/U) 34-mer oligonucleotide substrates by *E. coli* dsUDG, hTDG or its minimal active deletion mutant 5' Δ 112/3' Δ 50 (compare Fig. 1b). An autoradiogram of a 20% denaturing polyacrylamide gel is shown. b, Processing of U and G/U 90-mer substrates by UDG and dsUDG present in extracts of isogenic *E. coli* strains NR8051 and NR8052 in the presence (+) or absence (-) of the UGI peptide inhibitor. The band immediately below the U substrate is due to exonucleolytic degradation of the single-stranded oligonucleotide in the crude bacterial extracts; the autoradiogram is of a 12% denaturing polyacrylamide gel. c, Northern blot analysis of total *E. coli* RNA using the dsUDG DNA as a probe. Right, total RNA separated by agarose gel electrophoresis; abundant 23S, 16S and 5S rRNAs are arrowed. Left, autoradiogram of the membrane hybridized with a dsUDG-specific cDNA probe; arrow indicates the specific dsUDG band with an electrophoretic mobility corresponding to ~700 nucleotides.

FIG. 5 Uracil glycosylase activity in extracts of insect cells. The 90-mer substrates G/T, G/U and U were incubated with whole-cell extracts of human (HeLa) or insect cells. S2, Schneider's line 2 cells derived from 20–24-h-old embryo of *D. melanogaster*; Sf9 line derived from pupa of *S. frugiperda*; H5, line derived from embryo of *T. ni*. Lanes +UGI, reactions carried out in the presence of the peptide inhibitor UGI; autoradiogram is of a 12% denaturing polyacrylamide gel.



there is a need for an enzyme such as the highly conserved UDG protein which can remove the aberrant base; during rest or in non-proliferating tissues, only an enzyme capable of processing G/U mispairs arising from cytosine deamination, such as dsUDG, may be necessary. In pupating insects, it may constitute the only defence against this hydrolysis.

This enzyme has escaped detection until now because the G/U activity cannot be detected in the presence of the abundant UDG enzyme unless this is inhibited by UGI. Moreover, most previous studies made use of plasmid DNA uniformly substituted with UMP residues incorporated during *in vitro* DNA synthesis, such that the uracil was in A/U pairs, which are not substrates for the G/U-specific activity. In some experiments, plasmid DNA was treated with bisulphite to generate G/U mispairs by cytosine deamination (reviewed in ref. 16), but with low efficiency and giving uracils at random sites, making detection difficult. Synthetic oligonucleotides permit the construction of well defined substrates, matched and mismatched, single- and double-stranded, where the uracil is situated in 100% of sequences at a predetermined site.

Does deamination of cytosine present such a mutagenic threat that organisms have evolved a double protection to counter it? The antimutator effect of TDG has not been quantified as cells devoid of this activity are not yet available. The *E. coli ung*⁻ strains display a 30-fold higher frequency of C-to-T transitions than the isogenic *ung*⁺ parent¹⁷, which implies that UDG is responsible for a significant amount of G/U correction *in vivo*. The G/U-specific activity in bacterial extracts suggests that bacteria need this enzyme, which will be substantiated if the mutator phenotype of an *ung*⁻ strain augments upon inactivation of dsUDG.

We originally thought of TDG as a recently evolved enzyme that arose because of the inclusion of 5-methylcytosine into the DNA of higher organisms and that its principal role was in the repair of G/T mispairs arising from the deamination of this base¹. Our present findings indicate that TDG evolved from a prokaryotic G/U-specific enzyme which acts either as a backup or alternative to UDG in the repair of G/U mispairs arising through spontaneous hydrolytic deamination of cytosine. □

Methods

Nicking assays. These have been described^{1–3}. The protein amounts used were: purified *E. coli* UDG, 0.5 units; affinity-purified hTDG, 20 ng (2 units)⁵; total extracts, 10 µg; reticulocyte lysates, roughly equal amounts of *in vitro* translated proteins. Incubations were at 37 °C for 30 min for purified proteins, at 30 °C for 3 h for reticulocyte lysate mixtures, or for 16 h for total extracts. 50 fmol of oligonucleotide substrates (34-mers; 90-mers for total extracts) in the absence or presence of the UGI peptide (2.5 units) were used. Samples were treated with 0.1 M NaOH to cleave DNA at abasic sites before electrophoresis.

Construction of the TDG-deletion mutants. The full-length (FL) and the 5'-deletion mutant hTDG constructs were generated by PCR amplification of DNA fragments coding for the corresponding polypeptides (FL; 5'Δ25–264) using pHTDG plasmid¹ as template and the following oligonucleotides as primers (5' to 3'): FLs, GCCTCGGGCCATGGAAGCGG; 5'Δ25s, CATGCCATGGCTGAAGCTCCTAAT ATG; 5'Δ26s, CATGCCATGGCTCCAAAAGGAAGAAAAAGA; 5'Δ112s, CATGCC ATGGGTGTTTCAGAAGCTGAAGT; 5'Δ160s, CATGCCATGGCCAAAGTGTGTTTAT GTCA; 5'Δ233s, CATGCCATGGCCATTATGAAATTTTAGT; 5'Δ264s, CATGCC ATGGCCCTCTGCTATGTTATGCCA. The M13 reverse primer was used as the

antisense-strand primer. After *Nco*I and *Sal*I digestion, the fragments were inserted between the unique *Nco*I–*Sal*I sites of the pCITE-2b expression vector (Novagen) and the recombinants verified by sequencing. They were then linearized with *Sal*I and used as templates in *in vitro* transcription. To produce the C-terminal truncated polypeptides 3'Δ50–209, the full-length hTDG construct was linearized at the unique natural restriction sites *Ear*I, *Afl*II, *Xho*I and *Bgl*II. To produce the doubly deleted peptide 5'Δ112/3'Δ50, the 5'Δ112 construct was linearized at the natural *Ear*I restriction site. *In vitro* transcription and translation reactions were done using nuclease-treated rabbit reticulocyte lysate (Promega)⁴. 1-µl aliquots of the translation mixtures were analysed by SDS–PAGE followed by autoradiography (data not shown).

***E. coli* dsUDG expression.** The DNA fragment coding for *E. coli* dsUDG was obtained by PCR amplification using *E. coli* genomic DNA with the following oligonucleotide primers derived from the 5' and 3' regions of the *E. coli* ORF: Ps, CATGCCATGGTTGAGGATATTTGGCTCCAGGG; Pa, CCGTCGACTATCGCCACG-CACTACCAGCGCC. The ATG initiation codon was incorporated in an *Nco*I site with no amino-acid substitutions and a *Sal*I site was inserted immediately 3' of the stop codon to facilitate cloning. A single *E. coli* K-12 colony was diluted in 100 µl H₂O, boiled for 10 min and centrifuged for 10 min at 13,000 r.p.m. in an Eppendorf microfuge. 10-µl aliquots of clarified supernatant were used for PCR amplification in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂ and 100 pmol of each primer in 100 µl total. After denaturation for 5 min at 90 °C, dNTPs (200 µM each) and 1.5 units *Taq* DNA polymerase (Boehringer) were added. PCR was for 30 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min. After *Nco*I and *Sal*I digestion, the fragment was inserted between the only *Nco*I–*Sal*I sites of the pCITE-2b expression vector (Novagen) and verified by sequencing. *In vitro* transcription and translation reactions were carried out as already described.

Bacterial extracts and mRNA preparation. *E. coli* strain NR8051 and NR8052 protein extracts were prepared from 500 ml log-phase cultures¹. Total RNA from *E. coli* was prepared according to ref. 18 and 10, 20 and 30 µg were analysed by northern blot⁴ using random-primed labelled dsUDG DNA as probe.

Sequence alignments. The *E. coli* peptide sequence corresponds to an ORF on the *E. coli* K-12-genome (Genbank entry U28379; complement of bases 19462 to 20868). The *Serratia marcescens* amino-acid sequence corresponds to an ORF on the complementary strand of the 6'-N-acetyltransferase cDNA (Genbank entry M94066; complement of bases 722 to 1,210). The human (Genbank entry U51166) and mouse (PIR entry A46132) sequences are described in ref. 1.

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Going to work in genes catches on

Employment prospects are on the rise in genomics, genetic counselling and biotechnology. For the lucky few the rewards are great, but competition to break into the field can be tough. *Nature* reports trends from Europe and the United States on the eve of the American Society for Human Genetics meeting on 29 October.

Washington, DC. When asked why researchers in genetics have largely enjoyed the professional freedom of moving from academic research to industry, and sometimes back again, William Haseltine, chief executive officer of Human Genome Sciences (HGS), Maryland, and formerly chief of the AIDS research department at the Dana-Farber Cancer Research Center, Harvard Medical School, answers that his work in the industrial sector is not far removed from his work as a university professor.

The choice, as he sees it, was not between academic and corporate life; rather, it was a decision about how best to solve a problem. He states that "the university was not the right tool for the kind of scientific and biomedical discovery problems that I found interesting". In contrast to the university setting, Haseltine says that the large-scale, centrally organized genomics institutes make it possible to bring together the advanced technology and the many personnel necessary to solve some of the problems facing modern biomedicine. The creation of a genomics institute is not unlike the construction of the CERN supercollider, says Haseltine — it is merely a tool to

solve scientific problems. As examples, he cites his own institute in the industrial sector and the Sanger Institute (Hinxton, United Kingdom) in the government sector. "The driving force behind biomedical discoveries will be the ability to work with whole genomes at the bench and at the computer, together with the use of combinatorial chemistry and the use of statistical interpretation of large datasets," he continues. Information science will benefit the entire process.

Elke Jordan, deputy director of the NIH's National Center for Human Genome Research (NCHGR), seems to concur. She says that "in order to study interactions across larger numbers of genes in complex disorders, or between genomes, the skills of the computational biologist will be essential".

At Sequana Therapeutics in La Jolla, California, the company prefers to hire people who already have some experience in computational biology to design software and for database management. However, the explosion of biological information has made the specialist in the rapidly established discipline of "bioinformatics" a highly

that the growth of this field will depend largely on the comparative work yet to be done between the human genome and the genomes of other organisms (viruses, bacteria, yeast, nematode worm, mouse).

In a different approach to hiring policy, computer scientists at HGS have been recruited almost exclusively from the defence industry in the Washington, DC area, according to Haseltine. This recruitment strategy has been effective, he says, largely because of the experience of people with this background in handling and analysing large datasets. Haseltine suggests that, for much the same reasons, computer scientists with experience in climate modelling or financial analysis would also be well suited to cross over to the genomics field.

Interdisciplinary training

Joe Ecker of the University of Pennsylvania Plant Sciences Institute predicts that "for the next five to ten years the opportunities in bioinformatics will be great". He also believes that interdisciplinary training will be an important asset for people wanting to do genomics research. New masters' and PhD level programmes at the university reflect the multidisciplinary philosophy that is developing in the field of genomics. In the past, interdisciplinarity has not fared well for various reasons, not the least of which is the difficult treatment that grant proposals receive in peer review. In genomics, several research fields are coming together out of necessity; training programmes and funding mechanisms are responding in kind.

The University of Pennsylvania has initiated an interdisciplinary training programme in computational biology, focusing on applications of computer science, mathematics and statistics relevant to molecular biology and genetics, which is funded by a research training grant of the National Science Foundation. Students will be accepted into standard PhD programmes of either the Department of Computer and Information Science, or a biological department, but will then receive additional training in the complementary discipline. Postdoctoral fellowship positions are also available for those ►

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TABLE 1 INTERNET RESOURCES FOR MATERIALS USED IN WRITING THIS REVIEW

Genetics Societies/Organizations	http://
FASEB	www.faseb.org/genetics/
American Society of Human Genetics	www.faseb.org/genetics/ashg/ashgmenu.htm
The Genetics Society of America	www.faseb.org/genetics/gsa/gsamenu.htm
American Board of Genetic Counseling	www.faseb.org/genetics/abgc/abgcmenu.htm
National Center for Genome Research	www.ncgr.org/
The Institute for Genomic Research	www.tigr.org/
National Center for Biotechnology Information	www.ncbi.nlm.nih.gov/
Funding	
The National Center for Human Genome Research	www.ncgr.nih.gov/Grant_info/Funding/
NSF-Funded Training Program in Computational Biology	www.cbil.upenn.edu/UPCB/
Academic listing	
The Johns Hopkins University Bioinformatics	www.gdb.org/
Whitehead Inst for Biomedical Research/ MIT Center for Genome Research	www-genome.wi.mit.edu
Washington University Inst for Biomedical Computing	wucmd.wustl.edu/index.html
Lawrence Livermore National Lab's Biology and Biotechnology Research Program	www.llnl.gov/bbrp/
University of Pennsylvania Biology Department	www.sas.upenn.edu/biology/
<i>Arabidopsis thaliana</i> Genome Center	cbil.humgen.upenn.edu/~atgc/ATGCUP.html
Stanford Genomic Resource	genome-www.stanford.edu/
W.M. Keck Center for Advanced Training in Computational Biology	www1.pitt.edu/%7EJarzynka/keck/Frames/
W.M. Keck Center for Computational Biology in Houston, Texas	www.bioc.rice.edu/
Rutgers Computation Molecular Biology	cmb.rutgers.edu/
Industry listings	
Sequana Therapeutics	www.sequana.com/techplatmain.html
Base4 Bioinformatics	www.basefour.com/
Myriad Genetics	www.myriad.com/
Genetics Institute	www.genetics.com/
Molecular Information	www.molinfo.com/

sought-after commodity; consequently the demand outstrips the supply. According to Carlos Zamudio, a co-founder of Sequana, the company now has to compromise somewhat in its hiring practices, bringing a senior computational biologist together with a computer scientist, for instance, to train them until they can develop the fluency in biology they need to work more independently. Currently, the company has 45 researchers working in its bioinformatics division, of whom about two-thirds have PhDs and approximately 30 entered the company from outside biology research.

Since the company was founded in 1993, it has invested one-third of its budget in developing its computational resources. Zamudio feels

Mothers of the genome

LIKE many others who end up working in computational biology, Cathy Ball got into it by "accident". In 1992, a week after receiving her PhD in DNA recombination from the University of California at Los Angeles, she had her first child. Ball left her postdoctoral position at the University of California at Berkeley after only six months, as she was trying to work part-time and could not be as productive as she needed to be. She had heard from a friend that geneticists David Botstein and Michael Cherry were establishing the *Saccharomyces* genome database at Stanford University and were interested in providing more biological information than was previously available on existing yeast databases. The database project started out with one curator working ten hours a week,

has expanded to as many as five staff, but is currently at three. Historically, the laboratory's team of curators has been solely made up of women PhDs, all of whom have had young children. The team shares the responsibilities for the database, each working 15–30 hours a week. As the work is largely computer based, it is possible to work from the home if necessary. Like many, Ball's apprenticeship in computational biology has been mostly in the form of on-the-job training. Today, the group uses its expertise to introduce other researchers to the Stanford yeast database, as well as to the other resources to which the database is linked, such as Entrez, GenBank, the Yeast Protein Database and the Martinsried Institute for Protein Sequence (MIPS). □

who have demonstrated interdisciplinary research interests, particularly in areas such as physical and genetic mapping, biological databases, multiple sequence alignment, phylogeny construction, sequence search and analysis, simulation of control pathways, statistical methods, and discrete algorithms and combinatorial optimization in biology. (see Web site table previous page).

Ecker expects that a new masters programme in biotechnology, which is in development at the university, "will be able to take a biologist, and give them enough skills and knowledge to be useful as a bioinformatics specialist, but not necessarily a hard-core programmer". The masters programme will consist of three parallel tracks, molecular biotechnology, engineering biotechnology and computational biology/bioinformatics, the purpose being to encourage students to cross over into a complementary field that will expand their range of skills. At the same time, students will also be made aware of non-technical issues relevant to biotechnology, studying aspects of bioethical issues, government regulation, the drug-approval process and patent law.

Jordan says that at the NCHGR there is uncertainty about how best to train researchers in computational biology. She says that the Human Genome Program has been trying to facilitate cross-disciplinary training between biology and computer science, physics or engineering by encouraging people with a masters' degree in one of these non-biology areas to undertake PhD training. Some inroads have been made, Jordan says, but interdisciplinarity has not caught on as much as had been anticipated.

According to the NCHGR, most institutions have not, as yet, developed graduate and postgraduate training programmes in genomic science that would enrol students in molecular biology or one of the non-biological disciplines, yet also provide training to allow them to develop complementary expertise in another discipline. The NCHGR is offering financial support

for institutional programmes that are designed to train scientists with multi-disciplinary skills (more than one institution can apply as a consortium if it can be demonstrated that a well coordinated, integrated programme can be developed). The multi-disciplinary training programme in genomic sciences is intended to expand the research capabilities of individuals with backgrounds in either molecular biology or a non-biological scientific discipline relevant to genomics, for example, physical, chemical, mathematical, computer and/or engineering sciences.

Northern lights

In Canada there is not the infrastructure to operate on the scale of, for example, the United Kingdom's Sanger centre, and so the Canadians are in the process of building on their existing strengths in research. The Canadian Genetic Diseases Network, one of ten Canadian centres of excellence, was developed to promote advances in genetic research, to train Canada's young researchers and to create a partnership involving universities, medical centres, companies and Canada's Medical Research Council. This network comprises 38 of Canada's leading geneticists who are associated with eleven universities, eight hospitals and eight core facilities across the country. The network was founded in 1990 by Michael Hayden of the University of British Columbia, Vancouver. This "network of centres", as he calls it, provides funding for research and training opportunities, as well as an outlet for graduate students and postdoctoral fellows to employment opportunities with the network's industrial partners and spin-off companies.

The initial funding for training graduate and postdoctoral students came from the Network/Merck Frost award, which granted a total of CAN\$280,000 (US\$378,000) to graduate students and postdocs over the first four years of the programme. Hayden describes the network as a "centre without walls", where network sci-

entists can work in other laboratories in the network, learning specialized techniques of value to their own projects and bringing these skills back to their own laboratories. Such exchanges can be subsidized by a 'visiting researcher' fund, which provides limited support for up to 50 internode training exchanges annually.

Currently, the network is providing training for 70 postdocs, 86 graduate students, 21 research associates and 75 technicians (18 per cent of network participants are non-Canadian).

Because of the network's commitment to the transfer of technology to industry, students experience aspects of research and development, including patent and commercialization issues, that they might not encounter in a solely academic setting. "What we're doing is selectively filling the commercial infrastructure" so that the growth of these new ventures will provide opportunities for network trainees to put their skills into practice, says David Shindler, the managing director of the network.

Over the past two years, CAN\$300 million in early development capital has been raised for new commercial ventures from technologies stemming from research carried out within the network. Among these are the Canadian Medical Discovery Fund (CMDf), which totals CAN\$200 million, and the Neuroscience Partners Fund (CAN\$52.5 million), both of which are managed by MDS Health Venture Capital. As of mid-October the CMDf has made 19 investments to the tune of nearly CAN\$50 million, whereas the Neuroscience Partners Fund has completed 11 investments to date, in six Canadian and five US companies.

The dividend for Canada is the development of start-up biotech companies such as NeuroVir in Toronto, which was formally launched last month with CAN\$4 million of private financing. The company will focus on gene therapy for cancer and diseases of the nervous system. It hopes to raise a further CAN\$10 million over the next year.

Further financial and technical partnerships are planned with pharmaceutical and biotechnology companies in the field of cancer and neuroscience research. In the area of bioinformatics, the network will be launching new research and training initiatives to work closely with new Canadian bioinformatics companies such as a Base4, which was launched recently as a subsidiary of Allelix and is the first dedicated bioinformatics company in Canada.

The network also provides money to undergraduates for summer posts in network laboratories: in 1994–95, 46 Canadian undergraduates received funding for three-month training opportunities. An annual scientific meeting brings postdocs and graduate students together to take part in plenary sessions, brainstorming and career-enhancement workshops with industry representatives.

Brendan Horton

Counselling comes of age

Houston, Texas. In March 1969, 12-year-old Beth Fine read an article in the *New York Times* entitled *Will My Baby Be Normal?* In it, she learned of the then new prenatal technique of amniocentesis, which would enable doctors to detect possible birth defects in a fetus. She also learned of a new emerging field called genetic counselling and has been hooked ever since. Fine says that what struck her, even at such a young age, was "that if you could understand what the doctors were talking about, you could make decisions that were useful to you".

Now, more than 25 years later, Fine is a leader in the field, having entered it in 1979 and graduating with a masters degree in genetic counselling. She has been president

of the National Society of Genetic Counselors (NSGC) and is currently vice-president of the American Board of Genetic Counseling (ABGC), as well as head of the graduate programme in genetic counselling at Northwestern University Medical School, Chicago, Illinois. Sarah Lawrence College in Bronxville, New York was the first college in the United States to offer such a programme and took in its first batch of graduate students in 1969. Nationwide, there are now 22 accredited graduate programmes in genetic counselling.

As an indication of how the field has grown, Bea Leopold, executive director of the NSGC, says that when she joined the organization in 1987 there were 650 members; today, the membership has more than doubled to 1,450, all but 50 of whom are in the United States. Most states have at least one genetic counsellor, with the greatest concentration in California, New York State and Pennsylvania. However, a genetic counsellor's life can be a lonely and hectic one if, as in Idaho, Iowa, Mississippi and Wyoming, your catchment area is the entire state.

Most people enter the field with a BA or BSc in biology, genetics, psychology, nursing or a related discipline, and then go on to take a two-year masters-level graduate degree in genetic counselling. (For a list of accredited training programmes, contact the ABGC*). With only 100 to 125 new graduates entering the field each year, competition for slots is fierce. At 25, Sarah Lawrence College takes the greatest number of graduate students each year; next comes the University of Pittsburgh in Pennsylvania, with 12. More typically, the programmes take between four and six students.

Betsy Gettig, director of the genetic counselling programme at the University of Pittsburgh, says she receives about 120 applications each year for the 12 available slots. This year, she interviewed 55, all of whom were highly qualified on paper. With such bright students, it becomes "a very subjective thing", she says. But what undoubtedly helps her whittle the numbers down is whether people have had the initiative to visit a genetic centre and meet a genetic counsellor, or perhaps even 'shadow' a counsellor in their place of work. "That's the only thing that can separate the pool right now", says Gettig.

Those lucky enough to be selected will take a series of courses in common with other masters-level human geneticists and those on a PhD track. But what is unique to genetic counsellor trainees is the clinical rotation, which requires one-on-one supervision and can last anywhere between 12 and 20 months. As part of this on-the-job training students will see between 50 and 150 cases.

As programmes are often put together on a shoestring budget, funding for graduate training and/or scholarship money is almost non-existent. Most graduate students have to take out student loans, which "gets to be quite a financial pinch for these peo-

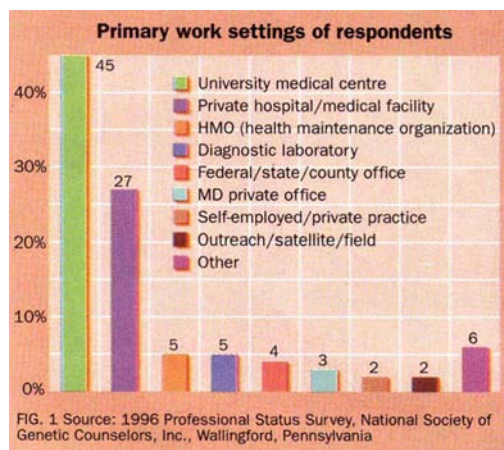
ple", says Gettig, as costs can run to between US\$15,000 and \$20,000 a year. People should shop around because tuition costs can vary wildly from region to region. Gettig says that her programme offers a small number of research assistantships, where tuition fees are waived and individuals receive a small stipend each month. But this appears to be more the exception than the rule.

Jobs: what's hot, what's not

In the past, most genetic counsellors were employed in the prenatal/paediatrics area. Although this is still the case, job openings are increasingly cropping up that are 'disease-specific'. This year's 'hot' jobs were in the field of cancer genetics, followed by neurodegenerative and psychiatric disorders. Even so, most genetic counsellors agree that, whereas graduates had the pick of the jobs a few years ago, those graduating in the past two to three years have had a tougher time finding employment, in part because of the downsizing that is occurring in the whole health-care sector as part of health-care reform.

Most graduating students, however, do find jobs without too much difficulty, particularly if their search is not limited to a specific region. The NSGC's JobConnection service may be a useful resource in this regard. A new addition to the pool of potential employers is the commercial laboratory, where genetic counsellors are being hired to help educate primary-care practitioners, as well as marketing and sales teams, about the tests being offered. Some genetic counsellors are also being recruited to provide over-the-phone counselling to individuals, or to act as the liaison between the commercial lab running the tests and the academic laboratories/medical facilities ordering the tests. It is also worth noting that this is a profession that is very amenable to part-time work.

With the kind of time pressures in a clinical setting, Gettig believes "it is unrealistic for the practising genetic counsellor to have much of a research emphasis". Although Gettig certainly describes the more typical experience, Barbara Biesecker hopes to be able to place a greater emphasis on research with the launch this year of a genetic

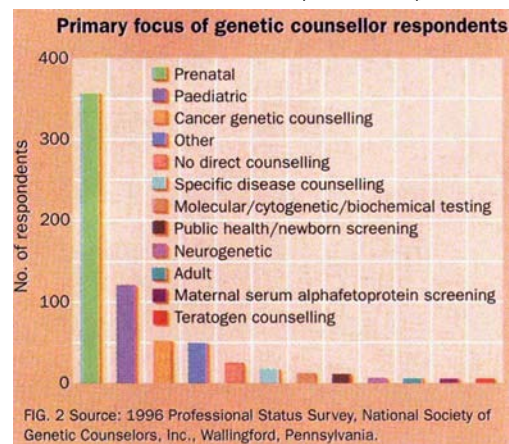


of the National Society of Genetic Counselors (NSGC) and is currently vice-president of the American Board of Genetic Counseling (ABGC), as well as head of the graduate programme in genetic counselling at Northwestern University Medical School, Chicago, Illinois. Sarah Lawrence College in Bronxville, New York was the first college in the United States to offer such a programme and took in its first batch of graduate students in 1969. Nationwide, there are now 22 accredited graduate programmes in genetic counselling.

Snapshot of the profession

So what does the profession look like? According to a recent survey conducted by the NSGC*, genetic counsellors are predominantly female (95% of survey respondents), Caucasian (93%), with a mean age of 36 years (23-70 range). Efforts have been made to attract more men and minorities into the profession but, as with so many other allied health professions, this continues to be an uphill struggle. Most genetic counsellors work in university medical cen-

*American Board of Genetic Counseling, Bethesda, Maryland (tel. 301-571-1825) and the National Society of Genetic Counselors, Wallingford, Pennsylvania (tel. 610-872-7608). Other useful contacts: American Society of Human Genetics, Bethesda, Maryland (tel. 301-571-1825) and the International Society of Nurses in Genetics, Fairfax, Virginia (tel. 703-698-3948).



counselling and research training programme at NIH's National Center for Human Genome Research, which is being run in conjunction with Johns Hopkins University. This programme will allow for further growth in the field to keep people stimulated, says Biesecker. Moreover, at a time when all aspects of the health-care system are coming under intense scrutiny, Biesecker says it is more important than ever for genetic counsellors to accumulate the data that demonstrate that what they do is meeting a need.

Biesecker, who combines teaching with research on the medical, behavioural and psychosocial aspects of gene testing in breast and ovarian cancer, nonpolyposis colon cancer and Marfan's syndrome, says she received 65 'qualified' applications for the four training slots available in this the first year that the programme has been operational. In the end, she says, the selection committee leaned more towards the 'older' applicants — those already with graduate degrees in a related field, those "who weren't intimidated by the fact that we were trying to change the field a little bit by training them to do research", and those willing to commute between the split teaching sites of Bethesda and Baltimore.

By and large, genetic counsellors seem to be a fairly contented lot. Dissatisfaction (where there is any) tends to centre on the issues of compensation and the lack of opportunities for upward mobility and career advancement. New graduates are quite well compensated: the average starting salary for entry-level counsellors is about \$35,000 per year. However, the problem has been one of limited upward growth potential, where the new kid on the block can be earning almost as much as someone with considerably more experience.

Salaries can vary from country to country state to state, and even within states. There are ways of boosting your salary, says Gettig, "you just have to be pretty creative". Gettig, who entered the field 15 years ago, worked in the trenches in the areas of paediatric, prenatal and teratogen counselling until three years ago when she was made programme director. Although this has given her the opportunity to teach, the trade-off has been a reduction in the amount of direct contact she has with patients.

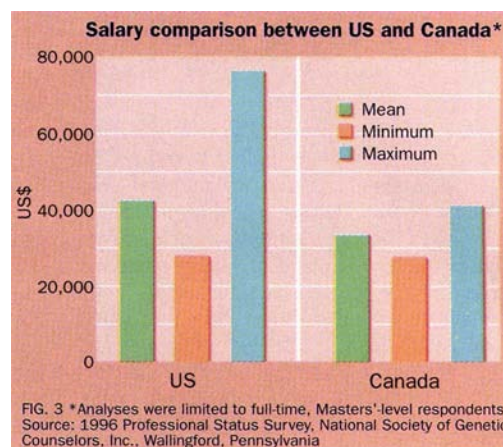
Then there is the question of recertification, which many feel is the final step to achieving full professional status. Although there is little economic incentive to be board-certified, says Gettig, according to the NSGC's survey, 70 per cent of respondents were certified by the ABGC. Most employers don't require it, although some are now asking that applicants be at least 'board-eligible', that is, have completed an approved masters'-level training programme.

The board exams are offered once every three years. For the first time this year certificates were 'time-limited' and counsellors will need to seek recertification in 10 years'

time. (Those certified before 1996 cannot be required to recertify, but will be encouraged to do so voluntarily.) Although most genetic counsellors agree that recertification is necessary to ensure a continued level of competence as new knowledge becomes available, many would prefer to see a system of continuing education credits introduced rather than a requirement to sit yet another exam. The ABGC is now considering its options.

The field of genetic counselling is clearly still evolving. Even though it can offer a real prospect for practical, preventive health-care, it is still unclear what effect changes in the health-care system, particularly the move towards 'managed care', will have on the delivery of such services. The question of reimbursement by insurance companies for genetic counselling is still unresolved.

Nevertheless, according to Fine, principal investigators are including provisions for genetic counsellors in their clinical research grants. Curiously, though, while there are



now graduate training programmes at St Mary's Hospital in Manchester, United Kingdom, and at Witwatersrand University in Johannesburg, South Africa, genetic counselling as a 'profession' is almost uniquely a North American phenomenon. But it has come a long way since the first class of ten genetic counsellors graduated from Sarah Lawrence College as part of the class of '71.

Diane Gershon

Tough competition in Europe

Throughout Europe, jobs for those with genetics skills are hard to find. But opportunities exist for good team-players in this expanding industry.

Oxford. The industrial application of molecular biology may be a recent phenomenon, but its impact on the number of jobs available has already been immense. Indeed, EuropaBio, the recently established pan-European biotechnology lobby in Brussels, representing both large and small companies, estimates that molecular biology underpins around 200,000 jobs in European industry. And with the changes in business focus that are currently taking place within the multinational pharmaceutical industry, coupled with the rapid growth of Europe's entrepreneurial biological sciences sector, it looks as if this influence will increase greatly in the next few years.

This situation has not gone unnoticed by Europe's politicians, who are becoming concerned about the growth in unemployment throughout the European Union as well as the quality of jobs now being created. "The European Commission has clearly recognised that biotechnology has the potential to create knowledge-based economies in the European Union. The Delors white paper [policy document] on European competitiveness acknowledged that biotechnology has significant potential for jobs growth," says Andrew Dickson, EuropaBio secretary general. Indeed, according to accountants Ernst & Young LLP (Palo Alto), the numbers employed in entrepreneurial US biotechnology companies grew from 40,000 to 108,000 between 1986 and 1996.

The numbers employed by Europe's entrepreneurial biological science sector have not quite reached the dizzy heights of their US counterparts — Ernst & Young's corresponding European report estimated that the sector is currently employing 17,200 people — but they are showing healthy growth rates of some 20 per cent per year as existing companies expand and new ones are formed. However, it is becoming increasingly clear that demand for jobs is currently outstripping new vacancies at rates of between 10 to 100 times.

Competition for jobs in molecular biology appears to be greatest on the European mainland. "We don't have any problems in hiring high-quality graduates and postgraduates. The problem is that there are just not enough jobs to go round in Italy," explains Glauco Tocchini-Valentini, director of the Institute of Cell Biology in Rome.

Consequently, many Italian scientists emigrate to where the jobs are, which frequently means the United States or to pan-European institutes such as the European Molecular Biology Laboratory (EMBL) in Heidelberg. And although Italian salaries for scientists are much lower at home than they could command either in the United States or elsewhere in Europe, Tocchini-Valentini is convinced that expatriates would return to Italy to work if the jobs existed.

A similar situation existed for molecular biologists in Germany until very recently

(see page 744). Political antagonism towards molecular biology and genetics during the 1980s and early 1990s created an employment vacuum for German molecular biologists. Biotechnology research and development was hamstrung by regulations, so scientists wishing to pursue careers in molecular biology had to move elsewhere.

After a long delay, German biotechnology is now establishing itself. The first biology start-up companies are confident they will succeed as they cherry-pick the best of German science both at home and abroad. "From a scientific point of view, the quality of scientists that can be hired is absolutely outstanding. In the German system people qualifying with PhDs at 28, 29 and 30 are much older than they are in the United Kingdom. They tend to be more mature and have a broader knowledge base," says Simon Moroney, the New Zealand-born chief executive officer of MorphoSys, the Munich-based combinatorial biology concern.

MorphoSys is going through a substantial growth spurt. It currently employs 20 people and plans to double in size by the end of 1997. "The sort of people we are looking to hire are scientists with a specific set of skills. It is much better to start with a very strong set of specific skills and broaden them later than start with a broad set and try to make them expert in one area. So we are looking for qualified molecular biologists, structural biologists, cell biologists, yeast geneticists and the like," adds Moroney. As for many other biotechnology companies, there is no attraction in recruiting graduates of the so-called biotechnology courses that were constructed in the late 1980s when universities saw what they believed would be an opportunity to attract new students.

MorphoSys is venturing into the new discipline of functional genomics — the attempt to assign the functional activity of a gene product to a sequenced gene as rapidly as possible — and is therefore looking to hire more people with a genomics background. "Such people are often geneticists that have specialized in one organism such as yeast, certain bacteria or mammalian systems, who also have the ability to take a screening technology and apply it." Most of the staff MorphoSys has employed are post-docs who have spent some time in the United States.

Recruiting homesick scientists is likely to be a major activity for many German biotechnology companies. Peter Heinrich, chief executive officer of MediGene, a Munich-based gene-therapeutic company, is finding the expatriate community an excellent source of new recruits as his company intends to grow from the current 26 employees to 40 by the end of next year. "Many excellent PhDs had to emigrate to the United States because of the lack of suitable positions in Germany. That situation is now changing and we want to attract them back," he explains. However, he adds, the door is not closed to other nationalities. "Earlier

this summer we had more than 100 applications in response to an advertisement in *Nature* for one position. The successful applicant was a Chinese postdoc who fulfilled all our requirements."

Molecular biologists hoping to walk into jobs with huge salaries and perks such as company cars need not look to the biotechnology sector. It is apparent that the entrepreneurs behind the new companies are looking for like-minded individuals. "We are not looking at candidates who are seeking secure jobs for the next 30 years and a fat salary. We want candidates who are entre-

preneurial, who have an understanding of what is involved and who are prepared to take a lower starting salary and share options," notes an executive at one UK biological science company.

Indeed, with so many skilled individuals seeking positions in these fast-growing technology-rich companies, executives can focus on other qualities in their recruiting. One crucial quality all biotechnology companies are looking for in potential recruits, as to a certain extent are multinationals, is the ability to be a team player. "That is a quality that can be hard to find sometimes in ►

Here come tomorrow's millionaires

London. Employment prospects for graduates in the life sciences are booming — and are likely to continue to do so for at least the next decade — says John Fulford, chairman of the Cambridge-based employment consultants Euromedica. "In terms of diversity of opportunity, we've never seen anything like it before."

According to Fulford, this is particularly true for those trained in molecular biology and related fields, as pharmaceutical companies turn towards 'rational' drug design, while the biotechnology industry, for which his company acts as a recruitment adviser, continues to expand at a rapid rate.

At the same time, says Fulford, the skills and aptitudes required of the modern life sciences graduate go far beyond those traditionally learnt at the laboratory bench. A key characteristic, for example, will be flexibility.

Part of this flexibility will lie in a willingness to move between regions, countries, and if necessary continents in search of opportunities. "Twenty or thirty years ago, a young graduate from, say, Manchester University would be loath to move far away," says Fulford. Today, in contrast, "one has to look at the global market place".

The converse is that, even in his or her home country, the life science graduate will face increasing competition from abroad. "Overall, we are going to see a shortage of skilled life scientists relative to the rapidly expanding global need. India, for example, is a marvellous repository of brilliant life scientists, and the Manchester graduate is going to be competing with young graduates from Bangalore."

Already the development of the biotechnology industry on a global scale is being affected by different national attitudes towards mobility. "British scientists and executives, for example, are about five times more mobile in their approach to employment than the average 'continental'," he says, suggesting that this may be linked to the historical tradition of working within the British Empire.

Adaptability will also apply to the choice of industry in which to work, given that, although there will be plenty of jobs, they may be in unusual fields. "A molecular biology graduate might consider the leisure industry to be the last area offering job opportunities," he says. "But a graduate from, say, the University of Bristol with a biology degree might become a specialist tour guide in Costa Rica."

A further skill that any graduate entering the biotech industry will require is communi-

cation. "This covers an ability to verbalize, to present thoughts in a logical manner, to listen, and to use modern technology to the maximum," says Fulford. Time spent on developing this skill — perhaps through a formal course — is time well spent.

Even the nature of jobs in the traditional pharmaceutical and healthcare sectors is likely to change dramatically. Both sectors, says Fulford, will continue to employ large numbers of graduates, but not necessarily in the traditional way. Many will no longer be employed by larger companies directly, but by smaller companies providing specialist services.

"Outsourcing is only just starting," he says. "A senior executive in the pharmaceutical industry told me recently that his company is questioning everything within its operation."

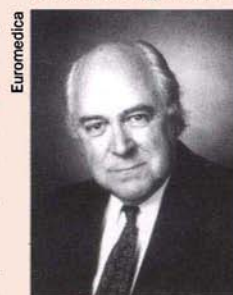
Are we the best people to manufacture our pills? Or to do our own research? Everything is subject to question."

This trend will shape the job market. Small 'start-up' companies will provide growing opportunities but, in contrast to the large pharmaceutical companies, will be looking primarily for specialist skills. "There is no room for the scientific generalist in these companies."

Again, flexibility is at a premium — particularly the ability to move between the lab bench, the accountant's office and the boardroom. "We have a great difficulty finding scientists who can move into commercial roles," says Fulford. Furthermore, he points out, moving into a small biotech company means working in "a highly insecure environment".

Yet the rewards, too, are potentially high. "People normally take the risk partly for the thrill and partly for the money," says Fulford, suggesting that even graduate recruits "should have the possibility of obtaining shares by six to 12 months of starting work". In particular, he points out, anyone joining a biotech company at an early stage has a great chance of doing well out of it. "Britain probably has more than 1,000 'biotechnology millionaires' — on paper, at least."

David Dickson



Fulford: flexibility is the key to success.

postgrads and postdocs because they may have worked on their own for so long," explains Andrew Sandham, president and chief operating officer of the recently established British functional genomics company, Hexagen (Cambridge, United Kingdom).

Like other biotechnology start-up companies, Hexagen has ambitions to develop a research critical mass as quickly as possible, growing from six employees this summer to 20 by the end of this year and between 35 and 40 planned by the end of 1997. Hexagen is looking to recruit scientists with strong fundamental skills and a talent for information technology. Being based in the United Kingdom means that Hexagen, in common with other local companies, will have an easier time attracting recruits with experience beyond academic research, although the relative closeness of gene-sequencing and bioinformatics institutes such as the Sanger Centre and the European Bioinformatics Institute (EBI) could be a fertile source of recruits who want to hone their skills in an entrepreneurial setting.

That is not to say that the institutes themselves do not have ambitious recruitment programmes. Paolo Zanella, director of the EBI, has marshalled the institute's rapid growth in the past three years from 20 to 80 employees and expects the head-count to rise to between 100 and 120 by the end of 1998. A mathematical physicist by training, Zanella is aware that recruitment policies should not be too scientifically exclusive. "The last time we advertised we asked for graduates and postgraduates in a relevant scientific discipline, such as information scientists, biologists, chemists, biochemists, but we would not exclude mathematicians or physicists as we believe they also could make a contribution," he says. "It really does not matter what their formal university education was if they are intelligent and are keen, as we can offer in-house training."

Zanella's needs are slightly different from his industrial counterparts, as the latter are seeking experts focused on one aspect of science whereas he is keen to attract multi-skilled individuals. "We need people who have a good knowledge of biology, information technology and information science. These are difficult to find, although the universities are beginning to introduce bioinformatics courses where students are taught how to handle databases, conduct similarity searches and which mix information technology/science with molecular biology."

EBI has three foci. Half its staff work in the services area, collecting sequencing data, inputting into databases and distributing that information. About a quarter are employed to carry out its in-house research, which tackles the problems associated with biological structure and function. The last quarter provides industry support, helping companies develop coherent bioinformatics activities. Most of the people recruited in research positions tend to be postdocs, while graduates and postgraduates populate the

EBI's service and support functions. Demand is fairly high, however. "We had more than 100 applicants for 10 positions we recently advertised," says Zanella.

Multinational pharmaceutical companies are also stepping up their own in-house molecular biology capabilities. The international pharmaceutical industry is in the process of significant consolidation. Companies are merging with each other as they attempt to lever out more value from their organizations. And although the numbers employed in research by the merged companies may not be increasing, the skills base and functions are changing. Many pharmaceutical companies are now recruiting more microbiologists than before, for example.

Earlier this year, Glaxo Wellcome (Greenford, United Kingdom), the world's largest pharmaceuticals company, launched a recruitment campaign to attract people who could bring new skills to the company's research capabilities. "We were looking for scientists who wanted to discover new drugs

faster than before, who were prepared to embrace new technologies and work in multidisciplinary teams," a spokesman explains. So the company advertised for immunologists, molecular virologists, molecular biologists and cell biologists, as well as for more conventional specialists in medicinal chemistry and pharmacology. From its advertisements in *Nature* and elsewhere, the company received 2,139 applications. It took on 35 new recruits.

Most pharmaceutical companies are reorganizing their research efforts so that there is more cross-fertilization of disciplines. Glaxo Wellcome has deliberately arranged its working environment at its Medicines Research Centre in Stevenage, United Kingdom, so that scientists from different disciplines cannot avoid working alongside each other. "Molecular biologists now meet and work with synthetic organic chemists — this is something that would not have happened in the past," says a company spokesman.

Mike Ward

Germans learn to bet on biotech

Munich. When Ernst-Ludwig Winnaker, head of Munich University's Genzentrum (Gene Centre), looks out of his office window, he sees the evidence of Germany's new biotechnology offensive. He views a huge building site that will extend the Gründerzentrum, a so-called incubator for new biotechnology companies. The German state of Bavaria is pouring DM28 million (US\$43 million) into this scheme.

Why are things suddenly in motion in Germany, where social forces have for so many years hindered the development of biotechnology? Restrictive laws have been relaxed, and public acceptance of genetics and biotechnology has slowly increased. The government is determined that Germany should catch up internationally.

Worldwide turnover of the biotechnology industry is expected to exceed US\$50 billion by 2000. The United States, reckoned to be ten years ahead of Germany, will contribute half of this sum. Germany has a lot of catching up to do. Analysts predict that its job market could double within five years. But that in the United States is also set to double — to 250,000 jobs. So the gap will widen.

If the biotechnology market is really going to increase dramatically in Germany, something has to be done to help new companies set themselves up. The biggest problem is lack of venture capital. The German government has now stepped in where private finances have traditionally feared to go. In spring it set up the European Recovery Programme (ERF), with a sum of DM1 billion, which will offer loans of up to DM15 million for small companies to develop and market innovative products.

The government has also increased its spending on technology-transfer programmes and increased research funds for

biotechnology by nearly 7 per cent — at a time when most research is being cut.

But things are also improving in the private financing sector. The establishment of two on-line stock markets should bring new funds to small biotechnology companies. Until now only well-established companies with at least three years' recorded profit were allowed on to the stock exchange.

However, according to the European Organization of the Self-employed, conditions remain bad in Germany for setting up new businesses, particularly because banks lack experts to advise on innovative technologies. Also, low-risk mentality continues to dominate in German society. As Jürgen Rüttgers, Germany's research minister, says, it is easier to raise capital on your grandmother's house than on a brilliant idea.

All this explains why there are around 1,300 biotechnology companies in the United States yet fewer than 100 in Germany. And, although Germany has approved the medical use of more biotechnologically produced pharmaceutical products than any other country, there are only six production centres.

If Germany were a gambling nation, punters would probably give low odds on the realization of Rüttgers' boast that Germany will become the biggest biotechnology force in Europe in the next decade. But there is no doubt that it will become at least a strong player in the field.

Winnaker is particularly optimistic. He would place his bet on Rüttgers coming through with his prediction. Bioinformatics is becoming much more important than sequencing technology at this stage in the human genome project, he believes, and at the moment no country has the lead. This is where Germany could shine. **Gabor Stiegler**